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A COMPARISON OF THE EFFECTS OF CERTAIN CORTICOSTEROIDS AND RELATED COMPOUNDS ON THE PRODUCTION OF CIRCULATING HEMOLYTIC ANTIBODIES IN THE MOUSE

I. STRUCTURAL VARIATIONS AT C-11 AND C-17[1]

BY SHIRLEY E. NEWSOM AND MARVIN DARRACH

Abstract

Seven crystalline C_{21} steroids with structural variations at C-11 and C-17 have been compared quantitatively for their capacities to inhibit the production of circulating hemolytic antibodies in the mouse. Four compounds were found to be active—cortisone, hydrocortisone, corticosterone, and 11-dehydrocorticosterone. Of these, corticosterone showed the greatest and 11-dehydrocorticosterone the least potency. Cortisone and hydrocortisone had approximately equal activities. The remaining three steroids, epi-hydrocortisone, 11-desoxycorticosterone, and 11-desoxy-17-hydroxycorticosterone, had no antibody-inhibiting effect.

Introduction

The production of circulating antibodies is one of the various biochemical reactions influenced by hormones of the adrenal cortex (1, 3, 4, 5, 6, 8, 9). Since antibody levels are accurately measured by quantitative immunochemical techniques, the activities of administered corticosteroids and related compounds can be compared by the observance of their effects on the production of antibody protein during immunization. Such information contributes to an understanding of the more general problem of relating the various biological activities of the steroids to their chemical structures.

Previous experiments have established that cortisone acetate suppresses the production of circulating hemolytic antibodies in the mouse and that corticosterone has a similar effect (8, 9). Additional C_{21} steroids have now been studied; this report describes the activities of those which differ from each other only at C-11 and C-17. They are as follows:

Δ^4-pregnene-21-ol-3,20-dione, *11-desoxycorticosterone* (DOC)
Δ^4-pregnene-17α,21-diol-3,20-dione, *11-desoxy-17-hydroxycorticosterone* (S)
Δ^4-pregnene-21-ol-3,11,20-trione, *11-dehydrocorticosterone* (A)
Δ^4-pregnene-11β,21-diol-3,20-dione, *corticosterone* (B)

[1] *Manuscript received August 5, 1955.*
Contribution from Department of Biochemistry, University of British Columbia, Vancouver, British Columbia.

Δ^4-pregnene-17α,21-diol-3,11,20-trione, *cortisone* (E)
Δ^4-pregnene-11β,17α,21-triol-3,20-dione, *hydrocortisone* (F)
Δ^4-pregnene-11α,17α,21-triol-3,20-dione, *epi-hydrocortisone* (EPI-F)

Methods

The chemical identity and purity of the crystalline steroids used in this study were confirmed by the following tests. Ethanol solutions of each compound gave absorption curves in the ultraviolet region with peaks within the 238–242 mμ range. Paper chromatography in toluene – propylene glycol or benzene–formamide (11) gave single zones as detected by both the absorption of ultraviolet light and the reduction of blue tetrazolium. Each steroid gave a characteristic absorption curve in sulphuric acid over the 230–600 mμ range (10).

The preparation of sheep erythrocyte antigen, the immunization schedule, and the quantitative comparison of immune hemolysins have been described in detail (8, 2, 7). The steroids were administered as single subcutaneous injections of fine suspensions in Tween 20 and saline at a concentration of 25 mgm. per ml. (9).

Numerous preliminary experiments were conducted with the seven steroids to detect activity and, if possible, to determine the doses causing partial and complete suppression of hemolytic antibody production. Since 5.0 mgm. of cortisone or hydrocortisone proved lethal to only a few animals, and 10.0 mgm. killed most of them within the duration of the experiments, the 5.0 mgm. dose was selected as a maximum for the comparison of the activities of the different steroids. If no inhibition of hemolysins resulted from an injection of 5.0 mgm. per mouse in two or more experiments, the compound was considered to be inactive. When almost complete suppression occurred with 5.0 mgm., lower doses of the steroid were employed in order to find a level which permitted the appearance of some hemolysins in the sera.

The following set of experiments was then conducted with the compounds under study to confirm earlier observations and to provide the data presented in Fig. 1.

On day 1, two groups of 10 Swiss albino female mice were injected subcutaneously with 5.0 mgm. of the steroid under test, and of those compounds that were active a 2.5 mgm. dose was injected into two other groups. A 1.0 mgm. dose was also employed when 2.5 mgm. gave complete suppression. On day 2, a standard dose of antigen was given intraperitoneally to these animals and also to two groups of 10 mice serving as non-steroid treated controls. On day 8, all animals were bled by cardiac puncture and the sera, pooled from appropriate groups of 10 mice, were later quantitatively analyzed for immune hemolysins. The strengths of the antisera, expressed as changes in optical density units per minute per ml. (O.D./min./ml.), were averaged for duplicate groups of 10 mice and the final results for the test groups were calculated to show per cent suppression of antibody from the level of the corresponding control groups.

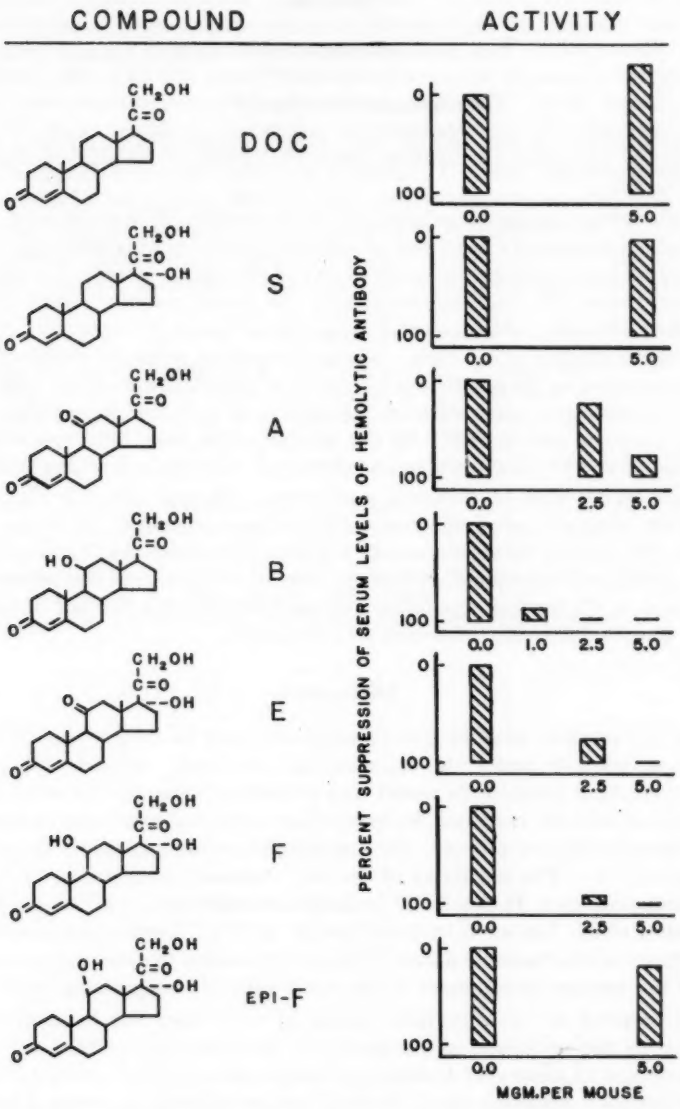

FIG. 1. A comparison of the effects of 11-desoxycorticosterone (DOC), 11-desoxy-17-hydroxycorticosterone (S), 11-dehydrocorticosterone (A), corticosterone (B), cortisone (E), hydrocortisone (F), and epi-hydrocortisone (EPI-F) on the production of circulating hemolytic antibodies in the mouse. Each bar represents data averaged from a group of 20 mice.

Results

As illustrated in Fig. 1, 11-desoxycorticosterone (DOC), 11-desoxy-17-hydroxycorticosterone (S), and epi-hydrocortisone (EPI-F) were inactive at the 5.0 mgm. dose. The slight elevation for 11-desoxycorticosterone and the slight inhibition for epi-hydrocortisone which appear on the chart fall within the normal variations encountered amongst control groups and are, therefore, insignificant.

Of the remaining compounds tested, corticosterone (B) was the most active; this steroid completely inhibited hemolysin production at a dose of 2.5 mgm. and exhibited considerable activity at 1.0 mgm. Cortisone (E) and hydrocortisone (F) had approximately the same potency, each showing complete antibody inhibition at 5.0 mgm. and great, though not complete, suppressive activity at 2.5 mgm. Cortisone acetate, when administered either as a suspension in Tween 20 and saline, or as Merck Cortone (8), completely inhibited hemolysin production at 2.5 mgm. and was, therefore, slightly more active than the free alcohol. Of the four active steroids tested, 11-dehydrocorticosterone (A) showed the lowest potency. The 2.5 mgm. dose gave only a slight suppression of doubtful significance, whereas the 5.0 mgm. dose inhibited hemolytic antibody production to approximately the same degree as did 1.0 mgm. of corticosterone or 2.5 mgm. of cortisone or hydrocortisone. In a subsequent experiment, when the dose of 11-dehydrocorticosterone was increased to 10.0 mgm., complete antibody suppression did not occur; the effect was similar to that observed with 5.0 mgm.

Discussion

The C_{21} steroids used in this investigation can be clearly designated as active or inactive under the experimental conditions defined. Of the six pure crystalline compounds tested and known to influence carbohydrate or mineral metabolism, cortisone, hydrocortisone, corticosterone, and 11-dehydrocorticosterone showed activity. Each of these has either a ketone or β-hydroxyl group at C-11. The inactivity of the two 11-desoxy compounds, 11-desoxycorticosterone and 11-desoxy-17-hydroxycorticosterone, and of epi-hydrocortisone, which has an α hydroxyl group at C-11, further emphasizes the importance of the chemical nature of the C-11 position in determining whether or not the compound will exert a hemolytic antibody-suppressing effect.

The presence of an α hydroxyl group at C-17 does not in itself impart activity to the molecule, as indicated by the fact that neither epi-hydrocortisone nor 11-desoxy-17-hydroxycorticosterone exhibited antibody-inhibiting properties. Although the 17-hydroxyl group in cortisone seems to enhance its activity over that of 11-dehydrocorticosterone, the presence of a 17-hydroxyl group appears to render hydrocortisone a less potent compound than corticosterone. Therefore, a clear understanding of the significance of the 17-hydroxyl as well as the 11-ketone and 11-β hydroxyl groups in the compounds studied must await further information concerning the metabolism of

the individual adrenal steroids in the mouse. Various conversions to active or inactive metabolites and the rates of these changes undoubtedly influence the over-all biological effect of any administered steroid.

Acknowledgments

This work was supported by a grant from the Division of Medical Research of the National Research Council of Canada. Grateful acknowledgment is also made to Dr. Augustus Gibson, Merck and Company, Inc., Rahway, New Jersey, for supplies of cortisone, hydrocortisone, corticosterone, 11-dehydrocorticosterone, 11-desoxycorticosterone, and to Dr. Merrill Speeter, The Upjohn Company, Kalamazoo, Michigan, for a supply of epi-hydrocortisone.

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A NOTE ON THE ENERGY OF ACTIVATION OF THE AMYLASE IN VARIOUS HUMAN BODY FLUIDS[1]

By Leslie Kovacs and Jules Tuba

Abstract

The activation energy was determined for the amylase present in the following fluids obtained from the human body: urine, duodenal fluid, saliva, and normal serum, as well as serum from patients with mumps, acute pancreatitis, and carcinoma of the head of the pancreas. Over a temperature range of 4°–37.4° C., with starch as a substrate, the value of the energy of activation was similar in all cases to that for bacterial α-amylase, and the mean value was 13,740 ± 200 cal./mole. Partial heat inactivation of the enzyme was evident in some cases at 37.4°. On the basis of the evidence obtained it appears that α-amylase is present in all the body fluids examined.

Introduction

Since the first demonstration of amylase activity in human serum in 1846 (4) there has been considerable discussion concerning the origin of the enzyme. It was subsequently observed that a rise in serum amylase activity accompanied certain pathological conditions such as acute pancreatitis, mumps, and kidney disease. Consequently a number of organs were considered to influence levels of the enzyme in serum, and discussions of this matter have been given by Somogyi (9) and Malinowski (5). Following studies of the cleavage products of starch and glycogen, Somogyi was of the opinion that the same amylase exists in blood serum, saliva, and pancreatic secretions. Subsequently, Meyer, Fischer, Bernfeld, and Duckart (6) showed that on the basis of crystalline structure and solubility the amylases from human saliva and pancreas are identical.

The origin of human serum amylase became of interest to us during a recent study of levels of the enzyme in surgical patients (3). Various body fluids from patients were available to us and the value of the energy of activation of the amylase in each was determined in an attempt to obtain information about the nature of the amylolytic enzyme in these fluids. Information of this nature was useful to Schønheyder and Volqvartz (7) in distinguishing pancreatic lipase and liver esterase.

Materials and Methods

Serum, saliva, duodenal fluid, and urine were obtained from an apparently healthy subject and stored in a refrigerator at 4° C. for periods generally not longer than two days. It was necessary to dilute the normal serum and duodenal fluid with an equal volume of 0.9% sodium chloride to approximate the enzyme level in undiluted urine. Saliva was diluted 1 : 40. Serum was

[1] *Manuscript received in original form June 27, 1955, and as revised, September 12, 1955. Contribution from the Department of Biochemistry, University of Alberta, Edmonton Alberta, with financial assistance from the National Research Council, Ottawa, Canada.*

obtained from a patient with mumps on the second day after the appearance of the parotid swelling, and this was diluted 1 : 10 with saline. The same dilution was necessary for serum from a patient with carcinoma of the head of the pancreas and from a patient with acute hemorrhagic pancreatitis. We also used α-amylase of bacterial origin (Nutritional Biochemical Co.) dissolved in saline (80 mgm.%).

Amylase activity was determined in the above fluids at temperatures ranging from 4°–37.4° C. Except with sera from the patients with mumps and with carcinoma of the pancreas where quantities were limited, the enzyme levels were determined by means of three different techniques, as a check on accuracy of our work. These methods, all of which employed soluble starch (Fisher Scientific Co.) as substrate, involved the estimation of enzyme activity by (*a*) the decrease in the starch–iodine color (2), (*b*) increase of reducing power measured with 3,5-dinitrosalicylic acid (1), and (*c*) increase of reducing power measured with alkaline copper and arsenomolybdate (10). The procedure was as follows. Into the reaction tube were placed 5 ml. of 0.0417 *M* phosphate buffer, pH 7.2, 2 ml. 2% starch solution, and 1 ml. of the enzyme-containing fluid. Following the addition of the enzyme, at times predetermined, to give the same amount of reduction at different temperatures, 0.5 ml. of the digestion mixture was removed for development of starch–iodine color *or* determination of reduction with the dinitrosalicylic method, and 6 ml. was removed for estimation of reducing substances by the copper–arsenomolybdate method. The time of incubation was varied from four hours at 4° C. to 12 min. at 37.4° C., and the reaction was found to be linear with time in all cases except at 37.4° C. The temperature of the water bath was maintained at 4° C. in the cold room, while higher temperatures were obtained by adjusting the thermoregulator of the bath in the laboratory. Amylase levels were determined in the sera from the patients with mumps and carcinoma of the pancreas by methods (*a*) and (*b*) above.

The enzyme concentration in the different biological fluids was first adjusted by dilution with physiological saline to an identical level of 40 Teller units per 100 ml. at 37° C. after 30 min. incubation. Next the activity of the enzyme in various fluids was measured by the above methods at 4° C. for four hours. Then at the different temperatures used above 4° C., the enzyme reaction was allowed to proceed for periods of time which permitted the same degree of amylolytic or saccharogenic activity. This eliminated the use of three different units or of conversion factors and permitted graphical representation on the same scale.

Results

The energy of activation for each amylase-containing fluid was essentially the same by each method employed. In each case logarithms of the reaction velocities (the reciprocals of the time of incubation) were plotted against the reciprocals of the absolute temperature, and the best-fitting line was drawn through the points. The lines for each fluid would have been practically

superimposed on the graph and consequently have been spread out in Fig. 1, on the ordinate. It was then possible to calculate the value of the activation energy, μ, from the Arrhenius equation, whence the slope of the line $= -\mu/2.303\ R$ calories.

In several instances the points at 37.4° C. fall below the line in Fig. 1, which indicates that some heat inactivation of the enzyme had occurred. The values of μ vary from 13,500 to 14,010 cal./mole, and the mean energy of activation is 13,740 $\pm$ 200 cal./mole. This differs from a previously reported energy of activation for human pancreatic amylase of 12,600 cal./mole (8), which was calculated at 20°–40° C. However, our temperature range was from 4°–37.4° C., and in most of the human body fluids we noted some inactivation at 37.4° C.

It would appear from our observations that the amylolytic activity associated with the human sources examined by us may be ascribed to α-amylase only (8). The extra enzyme found in the serum of patients with obstruction of the extrinsic ducts of pancreatic and parotid glands appears to be identical with the enzyme normally present in the serum. Therefore, both organs could be involved in maintenance of the normal levels of serum amylase.

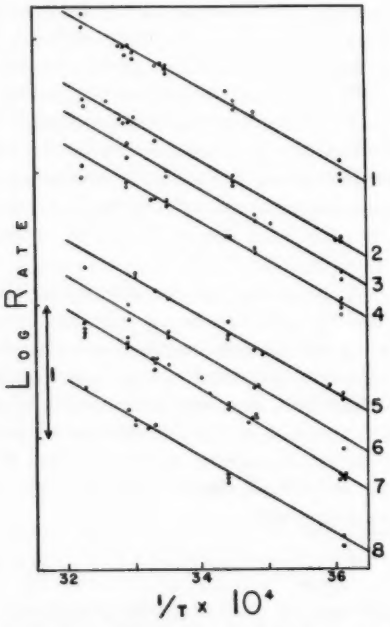

FIG. 1. Log rate of hydrolysis of starch by amylases from various human body fluids plotted against $1/T$. 1. Urine, 13.690 cal./mole. 2. Duodenal fluid, 13.630 cal./mole. 3. Serum (carcinoma head of pancreas), 13.550 cal./mole. 4. Serum (acute pancreatitis), 13.500 cal./mole. 5. Normal serum, 13.960 cal./mole. 6. Serum (mumps), 13.820 cal./mole. 7. Saliva, 14.010 cal./mole. 8. Bacterial α-amylase, 13.240 cal./mole.

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BIOCHEMISTRY OF THE USTILAGINALES

XII. CHARACTERIZATION OF EXTRACELLULAR GLYCOLIPIDS PRODUCED BY USTILAGO SP.[1]

By B. Boothroyd,[2] J. A. Thorn,[3] and R. H. Haskins

Abstract

A water-soluble compound formed by *Ustilago* sp. (PRL 627) in aerobic, submerged culture has been identified as D-mannopyranosyl-1-*meso*-erythritol. The extracellular "oil" produced at the same time by this fungus contains D-mannose, *meso*-erythritol, acetic acid, and a number of saturated and unsaturated fatty acids, probably as a mixture of D-mannosido-*meso*-erythritol residues to which the various acids are joined by ester linkages.

Introduction

The production and extraction of an extracellular "oil" formed when *Ustilago* sp. PRL 627 is grown in a medium containing cerelose have been described previously (2). Interest in the oil was aroused because of its high specific gravity (1.035 at 25° C.) and because it is formed in amounts as high as 15 gm. per liter of culture mixture. During studies on the chemical nature of this "oil", a second major product of the fermentation, a neutral water-soluble compound, was isolated. The characterization of this water-soluble compound will be described in this paper, as will the partial characterization of the "oil".

Concentration of the culture solution, after removal of the extracellular oil, cell material, and salts, gave a neutral, colorless, crystalline solid (7.3 gm., per liter). The solid, which chromatography indicated to be a single substance melted at 160°–161° C. after recrystallization from alcohol. Analysis indicated an empirical formula $C_{10}H_{20}O_9$. The substance contained carbohydrate, as shown by the anthrone test, but reducing groups were detected only after it had been heated with hydrochloric acid. Paper chromatography of the acid-hydrolyzate gave spots corresponding to D-mannose and *meso*-erythritol, and methanolysis of the compound allowed the isolation of methyl-α-D-mannoside and *meso*-erythritol. On oxidation with periodate, 1 mole of the compound ($C_{10}H_{20}O_9$) consumed 4 moles of periodate to form 2 moles of formic acid and 1 mole of formaldehyde. On the basis of these data, it is believed that the water-soluble compound formed in the fermentation is a D-mannopyranosyl-1-*meso*-erythritol. The configuration of the bond is probably β since the compound has an optical rotation of $-36.7°$.

Preliminary tests showed that the principal fermentation product, the extracellular oil, did not contain nitrogen, phosphorus, or sulphur, and that

[1] *Manuscript received August 29, 1955.*
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Issued as Paper No. 209 on the Uses of Plant Products and as N.R.C. No. 3779.
[2] *National Research Council of Canada Postdoctorate Fellow, 1952–54.*
[3] *Present address: Red Star Yeast and Products Co., Milwaukee, Wisconsin.*

it contained only a negligible amount of glycerol (0.2%, as determined by the method of Neish (6)). The oil gave a positive test for carbohydrate with the anthrone reagent, but free reducing groups were not present. Saponification values for different samples of the substance ranged from 245 to 312, with an average of 283. Free fatty acid was present in the order of 0.635 meq. per gm., and the oil contained about 3% of non-saponifiable material.

Saponification of the oil with alcoholic potassium hydroxide, followed by steam distillation of the acidified saponification mixture, led to the isolation of acetic acid (2.2 mM. per gm. of oil). The acetic acid accounted for 92% of the total acid collected in the distillate.

Methanolysis of the oil provided chloroform-soluble and water-soluble fractions. Distillation of the former (0.67–0.90 gm. per gm. of oil) indicated that it consisted of methyl esters of saturated and unsaturated fatty acids ranging from C_6 to C_{18} (and possibly C_{22}). No attempt was made here to collect methyl acetate. The water-soluble fraction (0.124–0.218 gm. per gm. of oil), upon crystallization, melted partially at 115° C. and finally completely at 165°–175° C. Fractional crystallization of the substance yielded α-methyl-D-mannoside. Chromatography on a Celite column, with a mixture of butanol, ethanol, and water as solvent, led to the isolation of both α-methyl-D-mannoside and *meso*-erythritol. The water-soluble fraction appeared to consist of only the two components, and its optical rotation indicated that they were present in almost equimolar amounts. It seems probable that these components exist in the extracellular oil as a mannopyranosyl-*meso*-erythritol.

The extracellular oil has been incompletely characterized, but the data indicate that it consists of D-mannopyranosyl-1-*meso*-erythritol residues to which are esterified acetic acid and higher saturated and unsaturated fatty acids. It is possible that some higher fatty acids also exist in the free state in the oil.

Experimental

Water-soluble Compound

A quantity of culture mixture was extracted with ether to remove the extracellular oil, and was then centrifuged. The supernate (2 liters) was mixed with methanol (1 liter) to coagulate cell material remaining after centrifugation, and the mixture was filtered through glass wool. The clear yellow filtrate was concentrated to 375 ml. and was then treated with animal charcoal. A portion of the resultant solution (150 ml.) was treated with Amberlite IR-120 and IR-4B resins to remove salts. The salt-free solution was then evaporated to a volume of 50 ml. and was again treated with charcoal before being concentrated to a thick sirup. Addition of ethanol to the sirup caused the formation of a solid (5.77 gm.) which, on recrystallization from 90% ethanol, gave colorless needles, m.p. 160°–161° C., $[\alpha]_D$ −36.7°. Analysis showed C, 42.17%; H, 7.073%. Calculated for $C_{10}H_{20}O_9$: C, 42.25%; H, 7.092%.

The crystalline material was subjected to paper chromatography, with *n*-butanol–ethanol–water (5 : 1 : 4) as solvent. Only one spot was obtained on the chromatogram. The material was then heated with a *N* hydrochloric acid for four hours, and a portion of the neutralized hydrolyzate was chromatographed on Whatman No. 3 filter paper with *n*-butanol–pyridine–water (6 : 4 : 3) as solvent. Two spots, having the same R_f values as D-mannose and *meso*-erythritol, were found.

These data indicated that the water-soluble compound consists of a molecule of D-mannose joined through a glycosidic linkage to a molecule of *meso*-erythritol. Further evidence for such a structure was obtained by oxidation of the compound with periodate. To 0.2883 gm. of the compound dissolved in 80 ml. of water was added 20 ml. of 0.4 *M* sodium periodate. After the mixture had stood at room temperature overnight, it was analyzed for excess periodate (3), and for formic acid (8), and formaldehyde (4). For each mM. of compound (calculated as $C_{10}H_{20}O_9$), 4 mM. of periodate had been consumed, and 2 mM. of formic acid and 1 mM. of formaldehyde had been formed. Such behavior would be expected from D-manno-pyranosyl-1-*meso*-erythritol.

Extracellular "Oil"

A 1.08 gm. quantity of the oil was refluxed with 40 ml. of 0.54 *N* potassium hydroxide and 7 ml. of water until the solution became clear. The mixture was then acidified to pH 1–3 with *N* sulphuric acid and was extracted with petroleum ether (2 × 100 ml.). Titration of the extracts indicated the presence of 0.47 meq. of acid. The extracted saponification mixture was steam-distilled until no further acid appeared in the distillate. Titration of the distillate showed the presence of 2.54 meq. of acid. The neutralized distillate was evaporated to dryness and the salts were dissolved in 10 ml. of 0.27 *N* hydrochloric acid. A 5 ml. aliquot of this solution was chromatographed on a column containing 50 gm. of Celite (5) and each effluent fraction was tested by titration with 0.01 *N* sodium hydroxide. Those containing the principal acid (92% of the total titer) were combined and evaporated to dryness. The sodium salt so obtained was converted to the *p*-phenylphenacyl ester, m.p. 109°–110° C., and to the *p*-nitrobenzyl ester, m.p. 77°–78° C. When mixed with the corresponding derivatives of acetic acid, the esters of the unknown acid showed no depression in m.p. Thus, acetic acid was the principal volatile acid obtained on saponification of the oil, and is present in the oil to the extent of 2.2 mM. per gm.

Titration of the ether extract of the residue from the steam distillation indicated the presence of 0.50 meq. of non-volatile acid, but the acid could not be demonstrated by the chromatographic method of Phares (7).

Methanolysis of the oil was effected by refluxing 9.45 gm. with 100 ml. of methanol containing 14.6 gm. of hydrogen chloride. After 18 hr. the reaction mixture was poured into 300 ml. of ice water and was extracted with chloroform (5 × 100 ml.). The aqueous residue was neutralized with silver carbonate, filtered, and the filtrate treated with hydrogen sulphide to remove excess silver

ions. After filtration, the solution was concentrated to a thick sirup which was induced to crystallize in the refrigerator by the addition of a small amount of ethanol. The white, crystalline material was filtered off, washed with a little alcohol, and air-dried. The yield was 1.66 gm., and a further 0.415 gm. was obtained by working up the mother liquor. The crystals did not melt sharply, but melted over the range of 115° to 175° C. Two spots were obtained when a solution of the crystals was chromatographed on Whatman No. 3 filter paper with the top layer of an *n*-butanol–ethanol–water mixture (4 : 1 : 5) as solvent. Ammoniacal silver nitrate was employed as the spray reagent. Separation into two components was found also when the crystalline material was chromatographed on a small Celite column with the above *n*-butanol–ethanol–water mixture as the developing solvent (periodate was employed to test the fractions). This system was then employed on a preparative scale. A 1.105 gm. quantity of the crystalline material was dissolved in 8 ml. of water, and the solution was placed on a column (25 × 3.4 cm.) consisting of 60 gm. of Celite and 80 ml. of water, with a top layer of 6 gm. of dry Celite. The butanol–ethanol–water mixture was then passed through the column. Nineteen 50-ml.samples of effluent were collected. Two distinct fractions were obtained, one occurring in samples 7 and 8, the other in samples 10 to 14. Recovery of the original material in these two fractions was 83%. The first fraction (0.490 gm.), after recrystallization from 80% ethanol, melted at 191°–191.5° C. and showed no depression of m.p. when mixed with authentic α-methyl-D-mannoside. Upon recrystallization from absolute ethanol, the second fraction (0.334 gm. crude) melted at 118.5°–119° C. Analysis showed it to have an empirical formula of $C_2H_5O_2$. The compound was neutral, showed no optical rotation, and had adjacent hydroxyl groups (periodate test). When mixed with authentic *meso*-erythritol, it melted at 118°–119° C.

Thus, the crystalline water-soluble material obtained upon methanolysis of the extracellular oil consisted of a mixture of α-methyl-D-mannoside and *meso*-erythritol. This mixture exhibited optical activity, with $[\alpha]_D = +48.6°$. Since the $[\alpha]_D$ of methyl-α-D-mannoside is + 79.2°, the mixture contained $\left(\frac{48.6}{79.2} \times 100\% \text{ or } 61.3\right)\%$ of the mannoside by weight. Thus, the mixture contained equimolar amounts of the two compounds.

In an attempt to determine the composition of the chloroform-soluble fraction obtained on methanolysis of the extracellular oil, 122 gm. of this fraction was subjected to distillation at 10 mm. pressure in a Podbielniak column (25 mm. × 90 cm.). The results of the distillation are shown in Table I. Methyl behenate was employed as a chaser to improve recovery of the C_{18} fraction. About 88% of the initial charge was recovered in the fractions. The iodine values show that all the fractions (except the first) contained unsaturated esters in various proportions.

The extracellular oil thus contains mannose, *meso*-erythritol, acetic acid, and a number of long-chain fatty acids, both saturated and unsaturated. Since the mannose and erythritol occur in equimolar quantities, it is quite

TABLE I

FRACTIONAL DISTILLATION OF THE METHYL ESTERS OF THE FATTY ACIDS FROM THE EXTRACELLULAR "OIL"

Fraction	Weight (gm.)	B.P. (° C.)	Iodine number	% of total	Published B.P. (1) (° C.)
1	6.356	43	.57	5.21	Methyl caproate 42° C.
2	4.142	43–135	9.77	3.39	
3	5.005	136	12.25	4.13	Methyl laurate 134° C.
4	3.537	136–159	59.30	2.9	
5	15.110	159–162	55.58	12.4	Methyl myristate 162° C.
6	3.204	162–177	58.70	2.63	
7	34.145	177–180	47.04	28.0	Methyl palmitate 184° C.
8	5.756	180–204	78.51	4.72	
9	26.842	204–207	104.6	22.0	Methyl stearate 204° C.
10	3.128	207–235	73.44	2.57	

possible that they exist in the oil as α-D-mannoside-*meso*-erythritol to which are attached acetic acid and the higher fatty acids. Since the extracellular oil contains some titratable acidity, it is possible that some acids occur in it in the free form and that the oil is a mixture of free acids and esters of mannosido-erythritol.

Acknowledgment

We are indebted to Dr. B. M. Craig for fractionations by distillation, Mr. J. A. Baignee for microanalyses, and to Mr. A. Funk for technical assistance.

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ADAPTATION OF THE CARBON DIOXIDE YIELD IN THE NINHYDRIN REACTION TO THE STUDY OF TRANSAMINATION IN PLANT TISSUES[1]

By F. S. Cook[2]

Abstract

A simple technique for assaying the glutamic–aspartic transaminase activity in plant tissues making use of titrimetric measurements of the carbon dioxide yield in the ninhydrin reaction with alpha-amino acids is described. The necessary apparatus is simple and inexpensive. The success of this method is verified by comparing results with those obtained on the same or similar reaction mixtures by quantitative paper chromatography, and by the chloramine-T method. Kinetic data on enzyme preparations of corn radicle homogenates and radicle segments frozen intact have been successfully obtained. The glutamic–aspartic transaminase is very active in corn radicles, giving high initial velocities. Per cent transamination per 10 min. period varies linearly with enzyme concentration. The optimum pH for the homogenates is at 8.5.

Introduction

The measurement of transamination activity, as of other enzymatic reactions which involve no exchange of gases, presents special problems, and since the discovery of transaminases by Braunstein and Kritzmann (3) considerable effort has been spent in the development of suitable methods for studying the properties of these enzymes. Various manometric techniques using the Warburg respirometer have been employed. Carbon dioxide can be released from glutamic and aspartic acids by specific decarboxylases isolated from bacteria (4, 24, 29, 34, 35, 44). Cohen (6) has converted glutamic acid to succinic acid, and measured the uptake of oxygen when succinic dehydrogenase is added. Oxaloacetic acid can be decarboxylated with aniline citrate (2, 7, 19, 24), and alpha-ketoglutaric acid has been non-enzymatically converted to succinic acid, and succinic dehydrogenase used for measurement of oxygen uptake (7, 19). Pyruvic acid can be measured colorimetrically (9, 28), and Tonhazy *et al.* (42) have used this method to estimate oxaloacetic acid by first converting it to pyruvic acid. Spectrophotometric measurement of the substrates is a useful technique for following the course of transamination reactions (5, 19, 30, 31), and is the only method available for obtaining reliable initial velocities. Recently many students have made use of the paper chromatogram for separating and estimating the amino acid substrates (11, 12, 13, 14, 15, 17, 18, 20, 32, 34, 35, 38). Tulpule and Patwardhan (43) have estimated the 2,4-dinitrophenylhydrazones of the alpha-keto acids on filter paper.

In 1940 Cohen (7) described a manometric method for measuring the activity of glutamic–aspartic transaminase using chloramine-T. This reagent

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can remove 2 moles of carbon dioxide from 1 mole of aspartic acid while removing only 1 mole from a mole of glutamic acid. The difference between the carbon dioxide produced by a reaction mixture at the beginning and at the end of a transamination period is a measure of the extent to which transamination has proceeded.

The method to be described here is similar to Cohen's chloramine-T method, but makes use of the reaction between ninhydrin (triketohydrindene hydrate) and alpha-amino acids. Ruhemann (39, 40) discovered that aqueous solutions of most alpha-amino acids when boiled with ninhydrin at a pH of from 1 to 5 evolve ammonia and carbon dioxide, and are changed to an aldehyde. Glycine does not form an aldehyde, but does evolve its carbon dioxide. The blue color of paper chromatograms of amino acids occurs above pH 4, and is the result of a product formed by ninhydrin condensing with a reduced form of ninhydrin and the liberated ammonia. Below pH 2.5 the color reaction does not occur, but carbon dioxide is quantitatively evolved. Van Slyke, MacFayden, and Hamilton (45) have developed a simple method for measuring this carbon dioxide, and using it as an assay for total alpha-amino acids in tissues. The reaction is carried out at 100° C. and under reduced pressure in a vessel on one arm of a U tube. When the reaction is complete the carbon dioxide liberated is distilled into the vessel on the other arm which contains standardized barium hydroxide solution. This solution is then titrated against a standard acid, and the amount of carbon dioxide can be calculated. These authors noted that under controlled conditions ninhydrin removes both carboxyl groups from aspartic acid, but only the alpha carboxyl from glutamic acid as does chloramine-T. Thus, difference in carbon dioxide production can again be used as a measure of transamination. The necessary apparatus is simpler and less expensive than that required for the manometric methods.

This method is restricted to the study of the following reaction:

$$\text{glutamic acid} + \text{oxaloacetic acid} \underset{b}{\overset{a}{\rightleftharpoons}} \text{aspartic acid} + \alpha\text{-ketoglutaric acid.}$$

Carbon dioxide that can be released from unit volume of digest by ninhydrin increases as reaction *a* proceeds, and decreases as reaction *b* proceeds.

Materials and Methods

Source of Enzyme

Terminal segments of the radicles of Golden Rocket hybrid corn were used in all experiments but one where whole etiolated seedlings were used. The seeds were soaked in water for one hour, and sterilized in a commercial solution of sodium hypochlorite (7% available chlorine) diluted with two volumes of water for an additional hour. They were then washed in sterile water, and planted out on sterile 2% agar in 6-in. Petri plates. The seeds were germinated in the dark at 30° C. for 70 hr. at which time the radicles were increasing in length at the rate of 0.95 mm. per hour. This sterilizing procedure eliminated

fungal and bacterial growth within the Petri plates. The radicles were excised by hand with a sterile blade reducing bacterial contamination of the preparations to a minimum.

In most of the experiments enzyme preparations were made by grinding the radicles with 0.2 *M* phosphate buffer of pH 8.0 in a glass homogenizer, centrifuging the suspension at a low speed, and dialyzing the supernatant against the phosphate buffer at 4° C. In a few cases intact radicle segments, quickly frozen, were used without further treatment.

System and Conditions of the Reaction

The transamination reaction is carried out in 25 ml. Erlenmeyer flasks with shaking at 38° C., and under aerobic conditions. To a flask is added either 4 ml. of a buffered homogenate or a number of frozen radicle segments and 3 ml. of the 0.2 *M* phosphate buffer of pH 8.0. To either enzyme preparation the following substrates are added: 1 ml. of 0.1 *M* L-amino acid, and 1 ml. of 0.1 *M* alpha-keto acid. The pH values of the substrate solutions are adjusted to 8.0 by the addition of potassium hydroxide.

Transamination is stopped by the addition of six drops of 6 *M* orthophosphoric acid, and 0.5 ml. of 10% sodium tungstate. This precipitates the protein, and causes the mixture to react acid to methyl orange indicator. After centrifuging the supernatant is transferred with washings to a boiling tube, and placed in a boiling water bath for two hours to reduce the volume, and destroy the keto acids. The clear centrifugate is made up to 10 ml. preparatory to analysis.

Analytical Procedure

For determination of amino acid carbon dioxide by the ninhydrin method 1.00 ml of this centrifugate and 1 ml. of water are added to a modified 25 ml. Erlenmeyer flask (45) containing about 50 mgm. of powdered citric acid–sodium citrate buffer, pH 2.5. The solution is carefully boiled over a small flame for a few seconds to drive off dissolved carbon dioxide, corked, and cooled to below 10° C. Carbon dioxide free air is passed through a second flask, while 1.00 ml. of 0.125 *N* barium hydroxide is added. When cooled the first flask is uncorked, and about 50 mgm. of ninhydrin are added. Both flasks are attached to a Pyrex glass U tube, and the apparatus is rapidly evacuated through a side-arm by means of an aspirator pump. Citrate buffer and ninhydrin are measured to within 10 % on small glass spoons similar to those described by Van Slyke *et al.* (45). Carbon dioxide free air is obtained by passing air from an airline slowly through a cleaning filter, and a soda-lime tube.

Five U tubes with flasks are prepared to this point, and then all are clamped in a rack, and placed in a boiling water bath for 12 min. The five flasks containing the barium hydroxide solution are then lifted over the edge of the tank into an ice-water bath, the reaction flasks remaining in the boiling water. Distillation is allowed to proceed with shaking for three minutes after which

the flasks are cooled, and carbon dioxide free air is allowed in through the side-arm. The remaining barium hydroxide is titrated against $N/35$ hydrochloric acid using 10 % phenolphthalein as an indicator. A 5 ml. burette with 0.01 ml. subdivisions is used.

All such determinations are made in quadruplicate, and with each set a blank is run containing only citrate buffer, ninhydrin, and water. The carbon dioxide evolved from a system after a period of transamination is compared with that evolved from an identical system in which enzyme activity is stopped at zero time by phosphoric acid and sodium tungstate. The milligrams of amino acid carboxyl carbon released from each system are calculated by subtracting the milliliters of hydrochloric acid required to neutralize the residual barium hydroxide from the water blank, and multiplying the difference by the factor 0.1715. With the system used here a difference of 0.10 ml. of $N/35$ hydrochloric acid is equivalent to 14 % transamination.

Comparison Experiments

Transamination in corn radicles was measured by quantitative paper chromatography and by the chloramine-T technique in order to verify the results obtained with the ninhydrin method. Aspartic and glutamic acids were separated by ascending paper chromatography using phenol–water as the solvent. The sheets were sprayed with 0.1% ninhydrin in *n*-butanol, the spots excised, and the amino acids measured by the method of Roberts and Frankel (36) using a Bausch and Lomb model 35 colorimeter, and a wave length of 575 mμ.

Reaction *a* and reaction *b* were shown by paper chromatography to proceed in the corn radicle at an appreciable rate. Fig. 1 shows a chromatogram

TABLE I

DETERMINATION OF GLUTAMIC–ASPARTIC TRANSAMINASE ACTIVITY IN CORN RADICLE HOMOGENATES BY QUANTITATIVE PAPEK CHROMATOGRAPHY

Recovery of total amino acid; 0.100 mM. aspartic acid added

	Aspartic acid recovered, mM.	Glutamic acid found, mM.
Reaction* stopped at zero time	0.099	Nil
Reaction time 30 min.	0.072	0.024†
α-Ketoglutaric acid omitted:		
zero time	0.104	Nil
30 min.	0.098	Nil
Boiled enzyme:		
30 min.	0.102	Nil

* *System and conditions of reaction: 4 ml. homogenate; 1 ml. 0.1 M L-aspartic acid; 1 ml. 0.1 M α-ketoglutaric acid. Temp. 38° C., pH 8.0.*

† *Per cent transamination: 24–28%.*

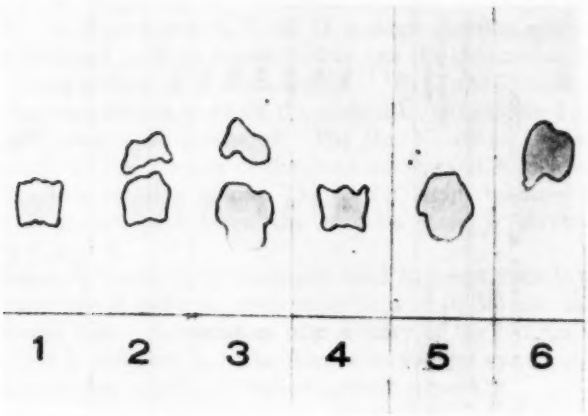

FIG. 1. Paper chromatogram showing the presence of glutamic–aspartic transaminase in corn radicles. Lower spots are aspartic acid; upper spots are glutamic acid. Complete system: 50 frozen radicles, 3 ml. phosphate buffer (pH 8.0), 1 ml. 0.1 *M*. L-aspartic acid, and 1 ml. 0.1 *M* α-ketoglutaric acid. Temp. 38° C.

1. Boiled enzyme.
2. Complete system after one hour incubation.
3. Same as (2) after phosphoric acid and sodium tungstate added.
4. α-Ketoglutaric acid omitted.
5. Reaction stopped at zero time with phosphoric acid and sodium tungstate.
6. Control spot of glutamic acid.

made from a series of reaction mixtures to which the substrates for reaction *b* were added, and with frozen radicles as the source of glutamic–aspartic transaminase. Table I presents quantitative data from a paper chromatogram of a similar experiment, but with a dialyzed homogenate as the enzyme source. When the reaction was stopped at zero time, when alpha-ketoglutaric acid was omitted, or when the enzyme preparation was heated in a boiling water bath for 10 min., no trace of glutamic acid was seen, and Table I shows that in each case all of the aspartic acid can be recovered. When transamination was allowed to proceed a spot of glutamic acid appeared (Fig. 1), and this glutamic acid plus the remaining aspartic acid accounted for 96% of the moles of amino acid added as aspartic acid (Table I). When aspartic acid was omitted from the otherwise complete reaction mixture using either frozen radicles or an homogenate as the enzyme source, no ninhydrin reacting substances appeared on the paper. From these results it was assumed that only transaminase causes the disappearance of aspartic acid, and therefore anaerobic conditions were not employed.

The ninhydrin method was also compared with the chloramine-T method of Cohen (7), and the results are shown in Table II. Q_T (N) is used as defined by Leonard and Burris (24), and is based on the nitrogen found in the precipitated protein of each reaction mixture. The centrifuged protein was transferred with repeated washings to micro-Kjeldahl flasks, and the nitrogen

TABLE II

A COMPARISON OF THREE METHODS USED IN THE DETERMINATION OF GLUTAMIC–ASPARTIC TRANSAMINASE ACTIVITY IN CORN RADICLES; HOMOGENATE COMPARED WITH FROZEN RADICLES AS AN ENZYME SOURCE

Experiment	Enzyme preparation	Method of analysis	Reaction time, min.	Direction of reaction	Protein N in system, mgm.	Per cent transamination	Q_T (N)‡	Per cent control recovery
A	Homogenate*	Chloramine-T	10	$\bar{a}$	0.58	21	—	—
		Ninhydrin				23	—	—
B	Homogenate*	Chloramine-T	30	*b*	1.3	29	44	105
C	Homogenate*	Paper chromatography	30	*b*	2.1	28	27	99
D	Homogenate*	Ninhydrin	30	*b*	1.1	28	53	110
E	Homogenate*	Ninhydrin	30	*b*	0.50	27	108	110
F	Homogenate*	Ninhydrin	20	*b*	0.83	29	104	109
G	Homogenate*	Ninhydrin	30	*b*	0.95	24	50	104
	Frozen radicles†					11	23	103

* *System and conditions of reaction as in Table I.*

† *Homogenate replaced by 3 ml. phosphate buffer (pH 8.0) and 100 6-mm. corn radicle tips.*

‡ $Q_T\ (N) = \dfrac{\mu M.\ amino\ acid\ transaminated}{mgm.\ protein\ N \times hours}$.

determined by an adaptation of the micro-Kjeldahl technique of Ma and Zuazaga (27). In Experiment A, Table II, a single reaction mixture and its control were analyzed by both the ninhydrin and the chloramine-T methods with results falling within 10 % of one another. B, C, and D show the results of three similar experiments in which the ninhydrin, chloramine-T, and paper chromatography assays are compared. The Q_T (N) values in these experiments are relatively low because of the large amounts of homogenates used, and the rather long reaction times. The Q_T (N) value becomes high when less protein is added, and when the reaction time is shortened as in Experiments E and F.

In Experiment G, Table II, an homogenate of 50 6-mm. radicle tips is seen to transaminate about twice as much aspartic acid in 30 min. as the same number of frozen tips. Nevertheless, the activity of the radicles when they are frozen intact is sufficient to make them a convenient source of enzyme in assays of transaminase activity in various growth regions.

Kinetics of the Corn Radicle Enzyme

That the carbon dioxide yield in the ninhydrin reaction can be used as an assay of transamination activity was substantiated further by employing it in certain kinetical experiments. An homogenate from etiolated corn seedlings catalyzed Reaction *a* at a rate about three times that of Reaction *b* (Table III). This agrees with the results of Albaum and Cohen with oat seedlings (1), but in corn radicles Reaction *b* will proceed to a greater degree than the 20 % reported by these workers (see Fig. 2). Reaction *b* was carried out in most of the studies reported here because of the greater ease of working with alpha-ketoglutaric acid than the unstable oxaloacetic acid. The time course of this reaction is shown in Fig. 2. Initial velocities are rapid, and the reaction rate tends to level off in about one hour. The curve is similar to those obtained by Cohen for preparations from pig heart (8), and by Albaum and Cohen for oat seedlings (1).

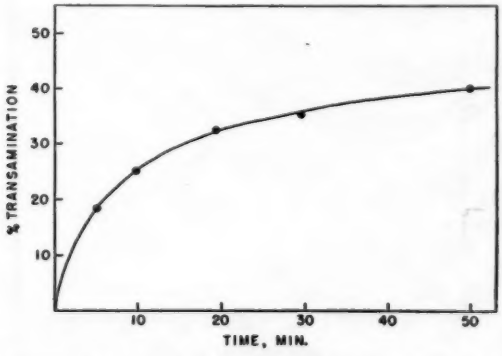

FIG. 2. Time course of the glutamic–aspartic transaminase Reaction *b* in corn radicle homogenates. System and conditions of reaction as in Table I.

TABLE III

A COMPARISON OF REACTIONS *a* AND *b* CATALYZED BY GLUTAMIC–ASPARTIC TRANSAMINASE

Reaction*	Reaction time, min.	Method of analysis	Per cent transamination	Q_T (N)
a	30	Ninhydrin	49	39
b	30	Ninhydrin	16	13

* *System and conditions of reaction: 4 ml. of homogenate of 4-day etiolated corn seedlings. Reaction a: 1 ml. 0.1 M L-glutamic acid and 1 ml. 0.1 M oxaloacetic acid. Reaction b: 1 ml. 0.1 M L-aspartic acid and 1 ml. 0.1 M α-ketoglataric acid. Temp. 38° C., pH 8.0.*

A straight line relationship exists between per cent transamination and enzyme concentration at low values of the latter (Fig. 3). It should be noted that in this graph the ordinate is expressed in per cent transamination per 10 min. period. Assays were made at the end of the 10 min. period only for the different substrate concentrations, and therefore initial velocities were not obtained. This linear relationship is convenient for comparing transamination activity in different tissues. Cohen (8) using pig heart preparations shows a straight line when per cent transamination per 30 min. period is plotted against the square root of enzyme concentration.

Reports of pH optima for transaminase activity vary from 6.9 to 9.0. Animal tissues usually give a value at or close to pH 7.5 (8, 10, 19, 22, 23). A variation of from 7.3 to 8.5 is reported for preparations from various bacteria (13, 21, 25, 37). Lichstein *et al.* (26), using partially purified preparations from *Streptococcus faecalis*, find that the rate of reaction of the

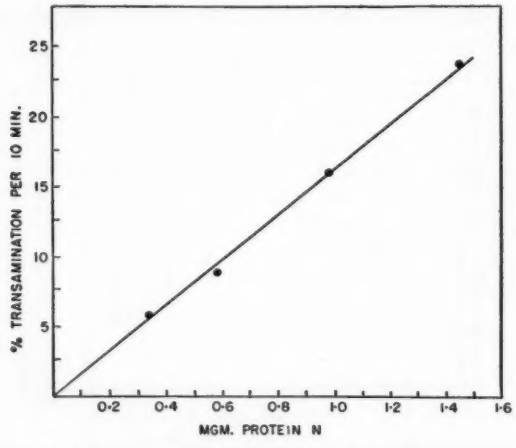

FIG. 3. Effect of enzyme concentration on glutamic–aspartic transaminase activity in corn radicles (Reaction *b*). System and conditions of reaction as in Table I.

glutamic–aspartic transaminase is only slightly dependent upon pH in the range 5.7 to 9.5. Fincham (14) has shown that in *Neurospora crassa* the pH optima vary with different transaminases: glutamic–aspartic, 9.0; alanine–glutamic, 5.5 to 6.0; and when isoleucine, ornithine, or phenylalanine are incubated with alpha-ketoglutaric acid, 8.0.

In higher plants the optimum pH for the glutamic–aspartic transaminase has been reported as 6.9 (33), 7.4 (16), 7.5 (41), and 8.5 (1). The glutamic–alanine enzyme of plants has a pH optimum of 7.4 or 7.5 (16, 17, 41), and the rate of reaction between gamma-methylene-glutamic acid and alpha-ketoglutaric acid is greatest at 7.4 (16). Fig. 4 shows that in crude homogenates of corn radicles the glutamic–aspartic transaminase has an optimum pH of 8.5 like that of oat seedlings (1). It is tempting to suggest that the enzyme of monocotyledons, or of the Gramineae, has this high value. However, Smith and Williams (41) report a value of 7.5 for seven different plants including barley, corn, and oats. At the present time it is difficult to account for this variation in pH optimum for one transamination reaction since many of the available data have been obtained from impure preparations from a variety of tissues, and we are dealing with an enzyme in which the relationship of activity to pH is somewhat complex because four ionizable substrates are involved. In the experiments reported here the systems were buffered at pH 8.0 in order that a phosphate buffer might be used.

These kinetic results along with the comparison experiments reported provided ample evidence that measurements of carbon dioxide yield in the ninhydrin reaction with alpha-amino acids could be successfully employed as an assay for glutamic–aspartic transaminase activity in the several phases of growth in the corn radicle. The results of such assays will be reported in a later paper.

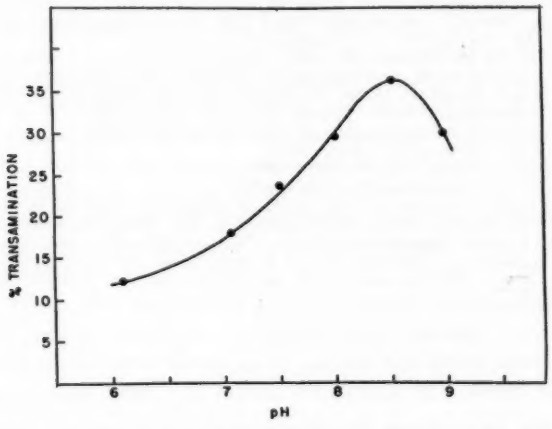

FIG. 4. Effect of pH on glutamic–aspartic transaminase activity in corn radicles Reaction *b*). System and conditions of reaction as in Table I.

Acknowledgments

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THE ALTERATION OF INTRACELLULAR ENZYMES

IV. KINETICS OF CATALASE ALTERATION INDUCED BY CHEMICAL AGENTS[1]

By J. Gordin Kaplan and Woon-Ki Paik

Abstract

The rate with which *n*-butanol alters the properties of yeast catalase has been studied as a function of temperature and concentration of altering agent. Activation energies for catalase alteration lay within the rather narrow range of 20–23 kcal./mole, thus confirming a prediction made previously on the basis of the difference in energies of activation for heat destruction of altered and unaltered catalases. Alteration by optimal concentration of butanol was a reaction of zero order. Chloroform also altered yeast catalase with an activation energy within this range of μ values. The close agreement in μ values leads us to conclude that the action of these two altering agents, at all concentrations, is characterized by the same rate-limiting step, even though their action differs in other respects. It was concluded that catalase alteration is probably all-or-none on the molecular level, rather than on the cellular level. Alteration was invariably accompanied by a decrease in the size of the treated cells; alteration was sometimes accompanied by changes in the cytochrome spectrum, but there was no causal connection between these two events. These data are consistent with the interfacial hypothesis, which, in its present crude form, pictures alteration as consisting essentially in the desorption of catalase from some intracellular interface at which it is normally bound in the intact cell.

Introduction

When intact yeast cells were treated with certain lytic agents (for example, chloroform and ultraviolet radiation), their catalase activity increased by well over an order of magnitude ((15), hereafter called Paper I); this increase in activity was called the *Euler effect* in honor of its discoverer (7). The Euler effect was accompanied by certain changes in the properties of yeast catalase that were considered to be due to some underlying change in the enzyme itself, rather than to extrinsic causes, such as, for example, a possible change in the permeability of the cell membrane. While an increase in permeability was not rigorously excluded as an explanation of the Euler effect itself, a good deal of evidence against such an explanation was adduced. The change in enzyme properties which accompanies the Euler effect has been called *enzyme alteration* (Paper I); our working hypothesis to explain it was that it consisted in the desorption of the enzyme from some intracellular interface at which the catalase normally exists (in a partially disordered condition) within the intact cell, the desorption being accompanied by increase in activity and altered enzymatic properties ((13) and Paper I). This is the *interfacial hypothesis.*

In Paper II (14), it was shown that the ability of homologous series of aliphatic compounds (alcohols, aldehydes, and ketones) to cause enzyme alteration (using this term hereafter to include the Euler effect) was closely correlated with their ability to reduce the energy at a model interface (air/

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water). Further, the critical concentration of altering agent (taken as that concentration required to cause doubled catalase activity of a standard yeast suspension) was independent of the concentration of the yeast cells. Both of these facts were considered to support the interfacial hypothesis, and the action of surface-active altering agents was considered to consist in deterging the enzyme from the interface at which it is normally held, in much the same way as these agents cause the breaking, or prevent the formation, of protein-stabilized foams (4).

In Paper III (9), a study of the effect of temperature on the kinetics of unaltered and altered yeast catalases showed certain significant differences between them. With respect to the enzyme–substrate reaction, it was shown that the experimental energy of activation (the μ of Arrhenius) was 4.5 kcal./mole lower for the altered than for the unaltered enzyme; the μ values were shown to be independent of substrate concentration. A possible permeability increase on alteration would of course have the effect of increasing the effective substrate concentration, but this could not account for the observed change in activation energy, since this does not vary with hydrogen peroxide concentration. Application of the theory of absolute reaction rates to these data showed that there was also a significant decrease after alteration in the free energy ($\Delta F^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation of the catalase – hydrogen peroxide system, of 1.7 kcal./mole and 10.1 entropy units, respectively. The decrease in the former was expected, following directly from (or causing (?)) the approximate 17-fold increase in activity upon alteration, whereas the decrease in the latter was not explained; its explanation may necessitate the introduction of a different activated complex for the altered enzyme – substrate compounds. Changes in the kinetics of the destruction of catalase by heat will be discussed in the Theoretical section, below.

The purpose of the present paper is to treat as a rate process the reaction of alteration itself, that is, to follow the transformation of the unaltered catalase of low specific activity into the altered enzyme of high activity and changed enzymatic and structural properties, and thus to test certain predictions made on the basis of the interfacial hypothesis.

Theoretical

In Paper I, it was shown that catalases altered by ultraviolet radiation and chloroform were inactivated by heat at a much greater rate than was the unaltered enzyme; we neglected to state in Paper I that the action of chloroform in increasing the heat-sensitivity of the cell enzyme could not be regarded as a direct action on the enzyme molecule itself, since the rates of heat inactivation for chloroform-treated and control solutions of catalase (e.g., crystalline liver enzyme) were the same.

In Paper III, this phenomenon was studied in more detail, and it was discovered that not only were the rates of inactivation at a given temperature different for unaltered versus altered enzymes, but the effect of temperature

on these rates was different for the two catalases, the altered intracellular catalase having an energy of activation (of heat destruction) approximately 21 kcal./mole higher and a $\Delta S^{\ddagger}$ 70 e.u. higher than the unaltered. On the other hand, the $\Delta F^{\ddagger}$ for heat inactivation of the altered enzyme was 2.8 kcal. *lower* than for the unaltered enzyme, accounting for the more rapid rate of heat inactivation at a given temperature.

The interpretation of the difference between the μ and $\Delta S^{\ddagger}$ of altered and unaltered enzymes, on the basis of the interfacial hypothesis, was that the latter, existing in a state of comparative disorder extended (partially) at an intracellular interface, required less energy to activate its heat destruction than the former; if the unaltered enzyme was already in a relatively disordered state, the formation of the activated complex for heat destruction should be accompanied by a smaller entropy increment than for the relatively more specific, altered enzyme. The assumption was thus made that the activated complex was the same for heat destruction of both altered and unaltered catalases.

On the basis of this interpretation of the heat inactivation data, we stated in Paper III that "the fact that the unaltered enzyme requires 21 kcal./mole less energy to activate its destruction indicates that bonds totalling 21 kcal. bond strength (perhaps four hydrogen bonds ?) must be formed during the process of enzyme alteration and this would therefore represent a minimum energy of activation for this process". The basis of this prediction is represented schematically in Fig. 1, where use is made of the picture of protein "denaturation" of Mirsky and Pauling (20) and Stearn (27), in which a large number of relatively weak bonds are broken during the activation step. The unaltered enzyme has less far to move along the reaction co-ordinate (i.e.,

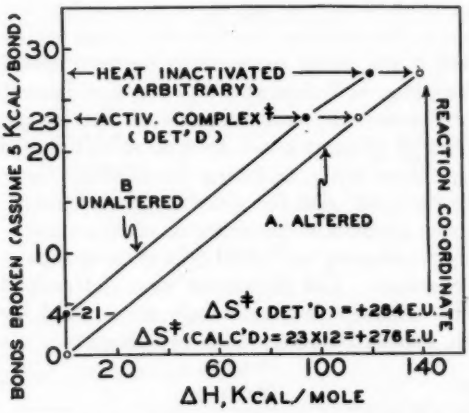

FIG. 1. Schema to illustrate value for energy of activation of catalase alteration predicted from activation energies of heat destruction of altered and unaltered enzymes. We assume arbitrarily that each bond broken has an energy of 5 kcal. and that total inactivation takes place when 30 such bonds have been destroyed.

towards heat inactivation) than has the altered, since four weak valences, present in the latter, do not exist in the former and hence need not be broken during the activation reaction. According to this view, these bonds must be formed during the alteration process itself.

It will be noted, incidentally, from Fig. 1 that the $\Delta S^{\ddagger}$ (for heat destruction of the altered catalase) calculated on the basis of an increase in entropy of 12 e.u. for each bond of 5 kcal. strength assumed to be broken during activation is quite close to the value actually measured kinetically (276 as against 284 e.u.); this supports the general view of the activation of heat "denaturation" put forward by Stearn (27).

Experimental

Methods of preparing, washing, and handling yeast cells were the same as those used in Paper II. All suspensions were prepared in phosphate buffer at pH 7.2. Incubations at different temperatures were carried out using the constant temperature bath and shaker previously described (Paper I), lower temperatures being reached by operating the bath and shaker, when necessary, in the cold room. In some of the experiments, treatment with altering agents was performed by using the refrigerated Warburg bath and shaker described in Paper III. Chloroform and *n*-butanol were dissolved in ethanol for use, as described in Paper II. Catalase activity was determined manometrically, as in previous papers. Duplicate activity determinations were made in all experiments.

The most difficult problem which we had to solve in order to carry out a study of alteration as a rate process was how to stop this rapid reaction after a given duration of treatment at constant temperature. A very successful method was devised, based on our finding in Paper II that, beneath a certain threshold concentration of altering agent, even an indefinitely long treatment caused no increase in the catalase activity of a yeast suspension. Thus, we treat the standard 0.9% yeast suspensions (as in Paper II) with altering agents for a given time, and then stop the reaction immediately by adding sufficient buffer to reduce the concentration of altering agent to below its threshold. A series of aliquots could thus be collected at different times of treatment, washed (three times, including the dilution) free of altering agent, resuspended in buffer, and used for activity assay, exactly as in Paper II. The Euler effect was adopted as the criterion of alteration.

Cell counts were made using unstained cells in the standard Levy hemocytometer counting chamber. Cell diameters were determined by means of a micrometer ocular. Absorption bands were measured by means of a Zeiss microspectroscope, calibrated before each experiment by means of a sodium lamp.

Results

Fig. 2 represents the course of change of catalase activity of the yeast suspension as a function of duration of treatment with *n*-butanol; each point is the average of four determinations, the standard deviations being shown.

It will be remarked that a straight line best fits the first points; the slope of this line may then be used as the rate of catalase alteration. This concentration of butanol, 7.63×10^{-1} M, had been found in Paper II to cause maximal alteration after an essentially infinite duration of treatment; it will be noted from Fig. 2 that at this concentration alteration was complete at room temperature in about one and one-half minutes.

The effect of temperature on the rate of alteration is shown in Fig. 3; alteration is seen to be quite temperature dependent within the range studied (5°–30° C.). Since all activity determinations were carried out at 30° C.

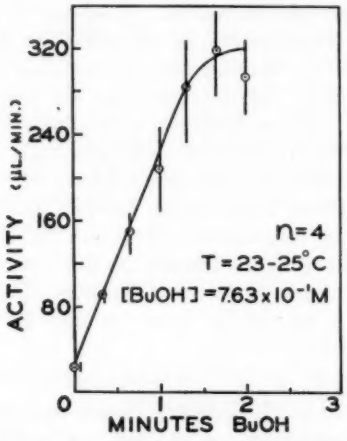

FIG. 2. Catalase activity of a standard yeast suspension as a function of duration of treatment with butanol. Standard deviations are shown; four determinations per point.

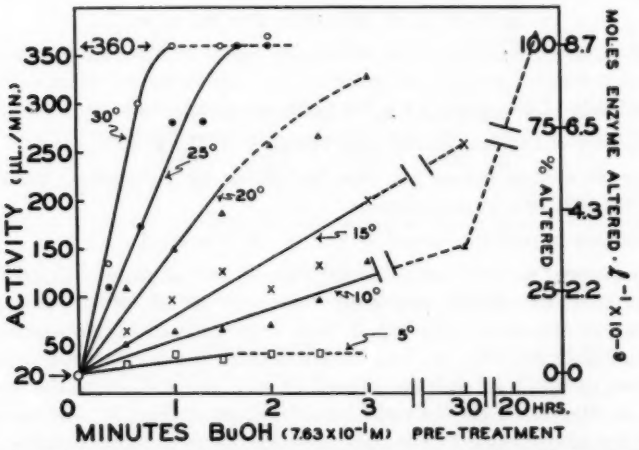

FIG. 3. Catalase activity of standard suspension versus duration of butanol treatment at temperatures from 5°–30° C. Assume maximum activity to be 360 μl./min.

regardless of temperature of butanol treatment, it will be observed from the 5° curve that heat subsequent to incubation with altering agent plays no part in the increase in catalase activity. Thus, our method of stopping the reaction seems acceptable. There was usually a good deal of scatter in the points, probably because of the rapidity with which sampling must be carried out, but at any temperature a straight line best seems to fit the initial experimental points.

It will be apparent from Fig. 2 and Fig. 3 (left-hand ordinate) that the zero-order rate constants for alteration (the slope of these curves) will have the dimensions of μl./min.^{2}, that is, an acceleration in the rate of appearance of O_2 following peroxide decomposition. However, it is possible to express these constants more meaningfully as concentration of catalase undergoing alteration per unit time, by assuming that the turnover numbers of crystalline beef-liver and of altered yeast catalases are the same. From Paper III, Table I, we obtain an average manometric turnover number for crystalline beef-liver catalase at 30° of 1.23×10^4 molecules H_2O_2/molecule catalase/sec. Since our present determinations were made with a substrate concentration 10 times that used in obtaining this turnover number (0.44 as against 0.044 *M* H_2O_2), we introduce the appropriate correction for this, and for the inactivation of catalase caused by substrate of this concentration (Paper III, Fig. 6), and obtain a corrected turnover number of 0.62×10^5 molecules H_2O_2/molecule catalase/ sec. for the liver enzyme. Let us write, from equation 21, Paper III,

[1] $$v_0 = 8.97 \times 10^{14} \cdot A \text{ molecules } H_2O_2/\text{sec.}$$

where v_0 is the initial rate of H_2O_2 decomposition and A is a pseudozero order rate constant whose dimensions are μl./min. It will be seen, from Fig. 3, that the activity of a fully altered suspension is approximately 360 μl./min.; substituting this value in Equation 1, we obtain

[2] $$v_0 = 3229.2 \times 10^{14} \text{ molecules } H_2O_2/\text{sec.}$$

Since the turnover number is, by definition, equal to this rate divided by the number of molecules present, in order to find this latter we simply divide the right-hand side of Equation 2 by the turnover number above to obtain:

[3] Molecules of catalase in reaction vessel $= 5.21 \times 10^{12}$.

To convert to moles catalase per liter, we divide by Avogadro's number and multiply by 1000 ml./l., and obtain:

[4] (Catalase) in reaction vessel $= 8.7 \times 10^{-9}$ moles/l.,

which are present to yield an observed rate of 360 μl./min. From Fig. 3 it is evident that the initial unaltered rate was 20 μl./min.; this activity represents zero alteration, and 360 μl./min. represents 100% alteration. From any intermediate activity, we may thus calculate the per cent alteration, and the number of moles of enzyme altered at any given stage of the alteration reaction, as illustrated by the right-hand ordinate of Fig. 3. Zero-order rate constants for alteration will thus have the dimensions of moles catalase altered per liter per second.

It is interesting to calculate the number of molecules of catalase per cell. In 1 ml. of the original 1% yeast suspension there were $24.4 \pm 5.0 \times 10^9$ cells, and 1/50th this number in the reaction vessels, 4.88×10^8 cells. Dividing this into the right-hand side of Equation 3, we obtain

[5] Number of molecules of catalase per cell $\leqq$ 11,000.

This value (N.B., for cells fully adapted to aerobiosis, but unaerated) depends, of course, on the validity of the assumption that the specific activities of crystalline beef-liver and altered intracellular yeast catalases are the same. That this assumption ought not to lead us too far astray is made clear by the similarity of Kat.f. values for beef-liver (30,000, Sumner and Dounce (28)) and for purified yeast (36,000, Brown (2)) enzymes; furthermore, other aspects of the enzymatic behavior of both are also similar (Papers I and III).

Rate constants for alteration obtained from experiments like that of Fig. 3 may be used for Arrhenius treatment, as shown in Fig. 4. It will be remarked that the Arrhenius equation is followed quite well, and this has been true of almost all our experiments. The μ value calculated from this curve is 22.6 kcal./mole; data concerning the effect of temperature on rates of alteration are summarized in Table I.

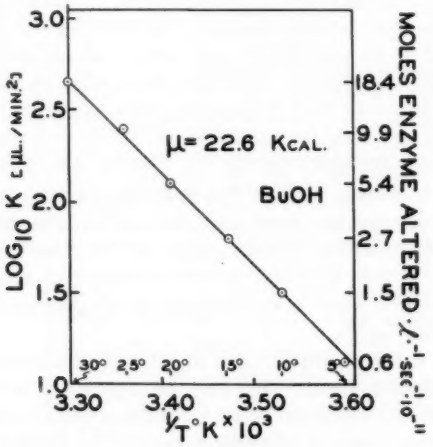

FIG. 4. Arrhenius plot of data of preceding figure.

TABLE I

EXPERIMENTAL ENERGIES OF ACTIVATION OF ENZYME ALTERATION; ALTERING AGENT WAS *n*-BUTANOL

Concentration of *n*-butanol	μ (kcal./mole)
A. 7.63×10^{-1} *M*	20.3
B. 7.63×10^{-1} *M*	22.6
C. 5.45×10^{-1} *M*	22.2
D. 4.36×10^{-1} *M*	23.5

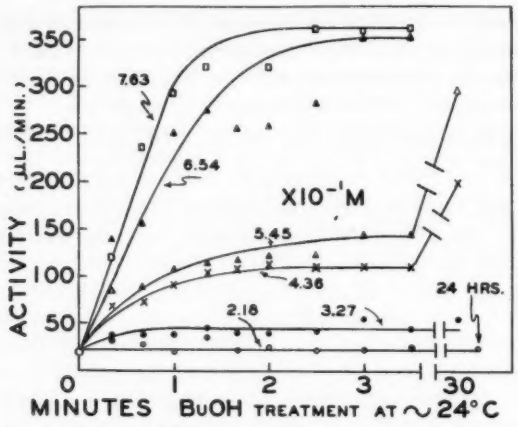

FIG. 5. Catalase activity of standard suspension versus duration of butanol treatment at varying butanol concentrations.

In Fig. 5 are plotted the initial rates of alteration for several different concentrations of butanol, the concentrations being those used in Paper II. With the lower concentrations of butanol, the initial rates of alteration were diminished, and at 5.45×10^{-1} *M* and lower, the final levels of activity attained were below maximal. At these lower concentrations of altering agent, the curves definitely look hyperbolic, and a case for an initially linear curve can be made out only for the highest concentration of altering agent. If the linearity of the alteration curves for 7.63×10^{-1} *M* butanol is due to a surface reaction, it is clear that with lower concentrations of altering agent the surface in question has not been saturated with the agent, and the rate of the reaction diminishes as the altering agent is "used up". It will be noted that with 2.19×10^{-1} *M* butanol, a concentration of altering agent more than one-fourth optimal, there is no evidence of enzyme alteration, even after a treatment of 24 hr.

In Fig. 6, Curve A represents the initial rates of alteration as a function of concentration of altering agent; this may be compared with Curve B, final catalase activities of the standard yeast suspension after prolonged treatment with varying concentrations of altering agent. The data for Curve B were obtained from the averages of 11 experiments, presented in line 2 of Table VI, Paper II. Curve A represents the averages of two experiments. There is a good correlation between the initial rate of alteration caused by any concentration of butanol and the final level of activity which it induces; the only real disparity is at 6.54 *M*, which is sufficient to cause the suspension to reach the maximal level of activity, but insufficient to cause it to do so at the maximal rate.

It was at this point necessary to see whether the temperature characteristics of "incomplete alteration" were the same as those reported above for optimal butanol concentration. The experimental energies of activation for alteration

by 5.45 and for 4.36×10^{-1} *M* are presented in Table I, lines C and D. It will be observed that the μ values were the same as those recorded for optimal rates of alteration, suggesting that the same reaction is rate-limiting in all cases.

In Fig. 7, Part A represents the rates of alteration induced by chloroform at three different temperatures. With this agent, our results were more variable and it was not possible to obtain several points intermediate between initial and final levels of activity, and consequently any rate constant for this reaction must be highly arbitrary. In order to determine an activation

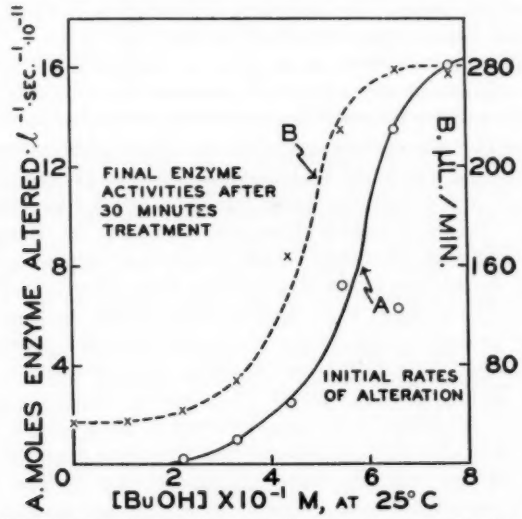

FIG. 6. A, initial rates of alteration, and B, final enzyme activities after 30 min. treatment, versus concentration of butanol. Data for Curve B, from Paper II, Table VI.

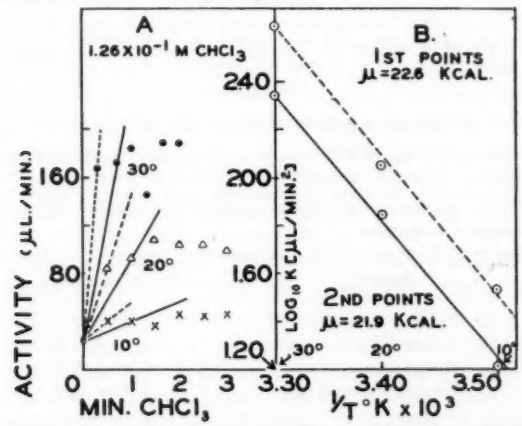

FIG. 7. A, activity versus duration of chloroform treatment, and B, Arrhenius plot of data of Part A.

energy for this reaction, we have simply taken as the rate constants the slopes of the straight lines joining the original point to either the first or second points along the curve, as illustrated in Fig. 7 *A*. When the effect of temperature on these somewhat capricious rate constants is represented in the Arrhenius plot, we obtain the curves shown in Part B of Fig. 7. The rates calculated from both first and second points give tolerably straight lines when their logs are plotted against $1/T$, and the μ values calculated from these curves are close to each other and to the values obtained previously with butanol as altering agent. In another experiment, a μ value of 19.4 kcal. was obtained for chloroform-induced alteration.

We should like also to present some data on the action of altering agents on certain other aspects of the structure and function of the yeast cell. All altering agents caused a decrease of about 10% in the diameter of the cells, as shown in Table II; the various agents did not differ in the extent of shrinkage produced. Freezing was found by Hansen and Nossal (11) to cause shrinkage of the yeast cell, and it will also cause the Euler effect (Kaplan, unpublished observations). This shrinkage is doubtless caused by the changed properties of both membrane and cytoplasm which permit a variety of substances such as nucleic acid derivatives (to be discussed in next paper in this series; however, see also (19)) to leave the cell, thus diminishing its osmotic activity.

Chloroform, propanol, and ultraviolet radiation also caused collapse of what Lindegren (18) calls the nuclear vacuole, when observed by the method of bright and dark phase microscopy (8), causing Fraser to speculate that catalase might be associated with this structure in the intact cell; Fraser also noted that the 'mitochondria', which prior to alteration appeared scattered on the periphery of this vacuole, were aggregated into clumps after altering treatments. Hansen and Nossal (11) also noted disappearance of the 'vacuole' as a result of freezing the yeast cells.

Since catalase is a member of the haem family, we investigated the effect of altering agents on the cytochromes, and some of these data are presented in Table III. Altering doses of ultraviolet radiation caused no change in

TABLE II

EFFECT OF ALTERING AGENT ON LONG AXIS OF THE YEAST CELL

Measurements of 102 cells of each category were made with micrometer eyepiece. Concentration of (or in the case of ultraviolet radiation, duration of treatment with) altering agent was sufficient to cause maximal catalase alteration. Standard deviations are shown.

Agent	Mean diameteters (μ)
A. None (controls)	5.4 ± 1.2
B. Butanol	4.9 ± 0.8
C. Chloroform	4.9 ± 0.7
D. Ultraviolet radiation	4.9 ± 0.9

Differences between means: A-B, A-C, A-D, highly significant at $P = 0.01$.

TABLE III

OBSERVATIONS OF VISIBLE SPECTRUM OF TREATED AND UNTREATED YEAST CELLS

(Position of β band unchanged)

Agent	Estimated center of α band of cytochrome				Increase in catalase activity
	a (or a')	b	?	c	
None (control)	606	565		555	No
n-Butanol	585		560		Yes
Chloroform	590	565		555	Yes
Ultraviolet radiation	604	565		555	Yes
Heat	585		560		No
Pyridine*	585		560		Yes
Acriflavin*	606	565		555	Yes

* *Applied directly to yeast on microscope slide for spectroscopic examination.*

the position of the alpha bands of cytochromes a, b, or c, chloroform shifted the a band towards the shorter wavelengths (a' (?)), whereas butanol not only shifted the a band, but also caused disappearance of the usual b and c bands and appearance of a single band located between the erstwhile b and c alpha bands (see Slonimsky (25, pp. 132–133)). Heat, which cannot alter catalase (Paper I), caused the same change as did butanol (see also (25)) and pyridine. Acriflavin, which like pyridine can cause a three- or four-fold increase in catalase activity (Kaplan, unpublished) had no effect on the cytochrome spectrum, despite the ability of this drug to change irreversibly the cytochrome content of the descendants of treated cells (the "petite colonie" mutant of Ephrussi (6)). It is thus evident that alteration may or may not be accompanied by a shift in the cytochrome spectrum, and that this latter may or may not be accompanied by alteration: there is no necessary relation between the two events.

Discussion

The experimental energies of activation of the process of catalase alteration induced by various concentrations of butanol and by chloroform lay within the narrow range of 19–24 kcal./mole. In Paper III, it was predicted on the basis of the interfacial hypothesis (a legitimate prediction, not made after the fact) that this process would have a μ of approximately 21 kcal.; this has obviously been borne out. We are more than somewhat embarrassed by the closeness of our actual data to the predicted value, since the latter was intended more as a rough estimate than as an exact predicted value, owing to the scatter in μ for heat destruction of unaltered and altered enzymes, from which data the prediction was made (Paper III, Table VI); the closeness of fit of predicted and determined values is thus doubtless a fortuity. Nevertheless, the temperature-dependence of alteration itself was quite reproducible. These data are therefore consistent with the interfacial hypothesis.

Also consistent with this hypothesis is the finding that alteration by 0.76 M butanol was apparently a zero-order process. If such zero-order kinetics were due to saturation of some other reacting molecule, rather than to a surface

reaction, the concentration of altering agent required to achieve a given degree of alteration would vary directly with the concentration of cells present in the suspension. However, it was shown in Paper II that the curves relating catalase activity to butanol or chloroform concentration were independent of cell concentration, thus eliminating the possibility of a stoichiometric reaction between altering agent and some unknown molecular species*. If the action of butanol and other surface-active altering agents, including anionic detergents, is to desorb catalase from some intracellular interface, the mode of action of chloroform, itself non-surface-active (see Paper II for discussion of this point; Mullins finds chloroform to be weakly surface-active (23)), is obscure. We have shown (Table III) that chloroform differs from butanol in its action on the yeast cytochromes and we shall show in the following paper in this series that it differs from butanol also in its effect on catalase structure. It thus seems likely, as we speculated in Paper II, that these two altering agents differ somewhat in their mode of action (see also (29) in this connection). Yet the similarity in the μ values of alteration suggests that the rate-limiting step is the same in both cases.

The fact that μ values for "partial" alteration (i.e., produced by less than optimal butanol concentrations) were the same as for complete alteration permits us to hazard the opinion that butanol-induced alteration is all-or-none per molecule (although probably not all-or-none per cell (Paper II)). Where a suspension possesses a catalase activity intermediate between the unaltered and fully-altered levels, it is probably because of heterogeneity within its population of catalase molecules, some, but not all, of which having undergone enzyme alteration. If, on the contrary, intermediate levels of activity were due to an incomplete alteration per molecule, one would not anticipate that a different reaction of this nature would be characterized by exactly the same activation energy as that of complete alteration.

Our figure of 11,000 molecules of catalase in the yeast cell may be compared to the 10,000–20,000 molecules of catalase per cell in *M* lysodeicticus in the study of Herbert and Pinsent (12). Their value was determined by comparing the Kat.f. of their crystalline bacterial catalase (incidentally, marking the first crystallization of a bacterial enzyme) with that of the lysed bacteria; since their estimate of catalase concentration per cell did *not*, we belatedly note, involve the use of intact cells, the critique of this estimate made in Paper I is entirely without foundation and must be withdrawn. These values may be compared with that of yeast cytochrome *c*, which we calculate from the data of Slonimsky ((25), Fig. 43, assuming that 15×10^{-19} moles cytochrome *c* per cell is the limiting value for fully adapted cells) to be 90,000 molecules per cell.

The number of molecules of catalase per cell will depend on the physiological state of the yeast culture, since Chantrenne and Courtois (3) and independently Slonimsky and Kaplan, working in Ephrussi's laboratory (26) have

* *Furthermore, we have found that the rate of butanol-induced alteration (7.63×10^{-1} M) is also independent of the cell concentration, over a 100-fold range (0.1% to 10% yeast), lending further support to our view that alteration is an interfacial phenomenon.*

reported that this enzyme, like the respiratory enzymes of yeast, increases greatly in amount as the cells are restored to aerobiosis after a period of anaerobic growth. The former authors, who assayed catalase in cell-free extracts, report a 20- to 90-fold increase in activity during aeration of a non-growing anaerobic culture, whereas the latter, assaying the activity of whole cells, reported only a threefold increase during aerobiosis. The explanation of this apparent discrepancy lies in the interesting fact that the Euler effect cannot be demonstrated in anaerobic cells (Kaplan, unpublished experiments). Thus, most of the activity of the newly synthesized enzyme cannot be detected within the intact cell, but is released upon lysis or extraction.

A similar situation was reported previously by Rickenberg (24) and Bonner (1) and by Sheinin and Crocker (personal communication) in the case of the celebrated enzyme of *Escherichia coli*, β-galactosidase. It has been known for many years that this enzyme can be caused to increase its activity after lysis of the adapted cells (5, 21, 17), and it was suggested in Paper I that this might be accompanied by enzyme alteration. That this is indeed the case has been shown in recent work done in collaboration with D. M. Bonner, in which the activation energy of this enzyme–substrate system was found to change after cell lysis in qualitatively much the same way as yeast catalase. This work will be published at a later date.

Butanol has been used by Morton (22) as a specific agent for the separation of certain enzymes from the insoluble formed elements of the cell. Morton believes that this action is due to the solution of the lipid components of such structures (mitochondria, etc.) in the alcohol, thus freeing the enzyme from the complex. Operationally, it might be difficult to establish a clear-cut distinction between dissolving away lipid from a protein–lipid complex, on the one hand, and deterging protein from the surface of lipid micelles (held, for example, by van der Waals forces operating between non-polar protein side chains and the lipid) on the other, especially when one makes use of solvents of pronounced surface-activity, like butanol. In any case, we have been able to create a model interfacial system (16, 10) in which crystalline catalase can be bound stably to the surface of a stabilized oil in water emulsion, and in which the enzyme can be deterged from the surface by octanol and other alcohols and surface-active agents with recovery of all of the properties of the soluble enzyme which had been modified while at the surface (i.e., recovery of full activity, and low μ for substrate decomposition). This is as yet a highly imperfect model of the intracellular catalase, but it is at least a step in the right direction.

We speculated in Paper I that some of the many cases of appearance of a particular enzymatic activity after cell lysis (e.g., utilization of substrates of the glycolytic and Krebs' cycles) might be due to an ***alteration in enzyme structure*** rather than to an ***unmasking***, in the sense of removing a physical barrier, such as a membrane, etc. However, it might be well to point out that here also there is a danger of the distinction between the italicized alternative being semantic rather than scientific, especially if the barrier is considered to be some internal obstacle, or steric factor, rather than the cell membrane.

The interfacial hypothesis, in its present naive form, attempts to explain too much and will require extensive pruning, or replacement altogether. Nevertheless, even if it subsequently develops that alteration is due to some quite different factor, this hypothesis will have been worthwhile, since we shall have had from its use additional knowledge about the intracellular state of catalase and of other enzymes.

The next in this series of papers will concern the effect of polychromatic ultraviolet radiation as an altering agent, and will adduce evidence that catalase exists within the cell in some sort of complex with ribonucleic acid or with its nitrogenous components.

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Note added in proof: *In calculating the number of moles enzyme per liter (Eq. 4), I stupidly neglected to consider the dilution of the cell suspension by substrate (total fluid volume in reaction vessel = 2 ml.). Eq. 4 should thus read:* (*Catalase*) *in reaction vessel* $= 4.3 \times 10^{-9}$ *moles/l., and right-hand ordinates of Figs. 3 and 4 and left-hand ordinate of Fig. 6 should be divided by 2. This fortunately does not introduce error into any of the other calculations presented. J.G.K.*

THE MUSCLE AND INTEGUMENT LIPIDS IN FEMALE ASCARIS LUMBRICOIDES[1]

By Donald Fairbairn

Abstract

Total lipids of the body wall (muscle plus integument) consisted of phospholipids (38%), unsaponifiables (8.1%), and triglyceride acids (47%). The phospholipid fraction contained major amounts of phosphoacetals, lecithins, and phosphatidylethanolamine. Sphingolipids and phosphatidylserine were probably present in smaller amounts. Much of the unsaponifiable matter was similar to, but not identical with, the ascaryl alcohol of the reproductive tissues, although sterols were also present. The triglycerides contained an approximately equimolar mixture of volatile and non-volatile acids. Phospholipid acids, on the other hand, were entirely non-volatile. The integument lipid fractions were similar in amount and kind to those of the muscle.

Recent reports from this laboratory have dealt with the lipids occurring in the reproductive tissues (5), embryonating eggs (6), and vitelline membrane (8) of the common pig roundworm, *Ascaris lumbricoides*. Among the unusual features of these lipids is the presence of considerable amounts of esterified volatile acids (chiefly acetic, pentanoic, and hexanoic acids) in the triglyceride fractions, and of a high molecular weight aliphatic alcohol (ascaryl alcohol) which is synthesized in the ovaries and subsequently incorporated into the vitelline membrane (8). Sterols are present in small amounts, the unsaturated sterol having been identified as cholesterol (7).

During the course of this work a few analyses were made on the lipids of the female body wall (muscle and integument) and on the muscle and integument (or cuticle) after separation from one another. Although no claim for completeness can be made, the results obtained are of interest because no similar description of the body wall lipids in a nematode has been published. In general, it can be said that the lipids of the female ascaris body wall are similar in kind, though not in amount or proportion, to those of the reproductive tissues. The integument, unexpectedly, was found to contain a full complement of the lipid fractions isolated from muscle.

Methods

Total lipids were extracted from two freshly isolated batches of body wall weighing 620 and 870 gm., respectively. In a third batch, the muscle (304 gm.) was stripped from the integument (69 gm.) with the aid of a steel spatula. The integument so obtained was thin and translucent, and free from visible particles of adhering muscle. Histological preparations of the integument appeared to be virtually structureless in unstained sections.

The extraction of total lipids, their separation into acetone-soluble (triglyceride and unsaponifiable) and acetone-insoluble (phospholipid) fractions, the saponification of these, and the subsequent determination of

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volatile and non-volatile acids, total unsaponifiables, ether-insoluble unsaponifiables and sterols, nitrogen, phosphorus, and iodine number have already been described (5). Reference to, or description of other procedures employed will be made where necessary.

Results

The pink, almost odorless body wall lipids were soft at 25° C. and almost wholly liquid at the normal body temperature of 40° C. Their gross composition in one of the two batches of tissue analyzed is detailed in Table I, which includes a comparison with the reproductive tissue lipids (5). Similar results were obtained from the second batch. It is seen that the total lipids of the body wall are about one-ninth as concentrated (per gm. wet weight) as the reproductive tissue lipids, and that the relative concentrations of the various fractions are much different in the two tissues. Thus, the reproductive tissues are rich in triglyceride fatty acids, which are laid down as food reserves for the embryonating and surviving eggs (6) whereas the body fats proper are not used during survival of the parasite *in vitro* (1). Nevertheless, the constitution of the reproductive and body wall triglycerides with respect to volatile and non-volatile acids is similar, for in both groups of tissues the weights of volatiles (mean molecular weight, 90) and non-volatiles (mean molecular weight, 290) are each sufficient to account for approximately one-half of the total triglyceride ester linkages. In the body wall, on the other hand, phospholipids are relatively abundant, as is the case in most muscle tissue. The very large unsaponifiable fraction in the reproductive tissues has already been shown (8) to be related to the need for ascaryl alcohol in the vitelline membrane.

TABLE I

LIPIDS OF THE FEMALE BODY WALL*

Fraction	Body wall		Reproductive tissues	
	% of wet weight	Relative concentration	% of wet weight	Relative concentration
Total lipids	0.68	100	6.05	100
Triglyceride acids				
(*a*) Volatile	0.060**	8.8	0.78	12.9
(*b*) Non-volatile	0.26	38	3.14***	52
Phospholipids	0.25	38	0.50	8.3
Unsaponifiables	0.055	8.1	1.29	21

* *By analysis of 620 gm. of tissue (149 gm. dry weight).*

** *Calculated from titrimetric data assuming the mean molecular weight to be 90, as is the case in reproductive tissue.*

*** *The phospholipid fatty acids which are included here do not introduce a serious error.*

The phospholipid and unsaponifiable fractions of the body wall lipids were examined in more detail. It was reported previously (5) that the reproductive tissue lipids were only faintly positive to the Schiff test (9) for phosphoacetals, whereas the body wall lipids reacted intensely. In the present investigation it was observed that saponification of the phospholipid fraction from body wall yielded fatty acids averaging about 50% of the initial weight of material instead of approximately 70% as required by theory for a mixture of lecithins and cephalins containing C_{18} acids. It was found that this discrepancy was accounted for if the phosphoacetals in the mixture were decomposed by treatment with dilute acid and the residual phospholipids separated from the free aldehydes by acetone precipitation.

The procedure used was as follows. The phospholipids (0.941 gm.) were emulsified in a mortar with a little water. The emulsion (10 ml.) was then shaken for one hour at 30° C., after which 0.13 ml. of 12 *N* hydrochloric acid was added, and the shaking continued for five hours. Twenty milliliters of petroleum ether (b.p. 30°–50° C.) was added and the mixture shaken. The stable emulsion was then broken by addition of 10 ml. of ethanol, and the mixture was centrifuged. After removal of the petroleum-ether layer the extraction of the aqueous layer was repeated. As some acid hydrolysis of phospholipids was anticipated in addition to complete hydrolysis of phosphoacetals, the combined extracts were shaken twice with 0.1 *N* sodium hydroxide to remove fatty acids, and were then dried and weighed. The alkaline extract was acidified, and the free acids dried and weighed. In this way 0.712 gm. of mixed phospholipids and aldehydes, and 0.074 gm. of fatty acids, were recovered. The latter represented some 0.106 gm. of phospholipids, or 15% of the acetal-free phospholipids. The phospholipid–aldehyde mixture was fractionated by solution in 12 ml. of petroleum ether and addition of the solution dropwise with continual mixing to 80 ml. of dry acetone. Precipitation of the phospholipids was completed by addition of two drops of a saturated ethanolic magnesium chloride solution. The precipitate was then dissolved in petroleum ether and washed with water. Yield, 0.547 gm. The acetone-soluble material was dried, dissolved in ether, washed with dilute alkali, and finally with water. Yield, 0.145 gm. of soft, solid, and faintly yellow aldehydes which gave a distinct Schiff test in a final concentration of 1 μgm. per ml.

Acetal-free phospholipids were analyzed for labile phosphorus (13) (lecithins and cephalins), total phosphorus, nitrogen, choline (4), fatty acids, and ethanolamine and serine. The latter two analyses were performed qualitatively by filter paper chromatography using butanol – acetic acid – water (4 : 1 : 1) as described by Pollak and Fairbairn for amino acids (12), with ninhydrin as the color reagent. Phospholipids were prepared for chromatography by heating for five hours in 6 *N* hydrochloric acid at 100° C. The solution was then cooled, extracted with ether, and the aqueous residue dried *in vacuo* over sodium hydroxide. R_f values for pure serine and ethanolamine were 0.24 and 0.46 respectively, with serine producing much the more intense color by reaction with ninhydrin. The hydrolyzed phospholipids gave

two spots on the chromatogram, with R_f values as above, but with the ethanolamine spot being intense and the serine spot faint. These results indicated, therefore, that very little phosphatidylserine was present in the body wall.

Complete results of analyses on the phospholipids, for each of the two batches of body wall examined, are presented in Table II. Phospholipids comprised about two-fifths of the total body wall lipids, and phosphoacetals comprised nearly one-third of the phospholipids. The remaining acetal-free phospholipids were mostly lecithins and cephalins, as judged by their lability in *N* sodium hydroxide (13), their large choline and ethanolamine content, and a N : P ratio approaching unity. The same data suggest that more alkali-stable compounds, such as the sphingolipids, were possibly present (as they are in most muscle tissue) in smaller amounts. No direct tests were made for such diaminophospholipids. Fatty acids were obtained from the purified phospholipids in approximately theoretical amounts (70%). They were distinguished by a very small iodine number, indicating a degree of unsaturation which is abnormally low by comparison with phospholipids from other sources. Unlike the triglycerides, the phospholipids contained no volatile acids.

Aldehydes isolated from the phosphoacetals were free from acids and phospholipids, as shown by their insolubility in dilute alkali and by the absence of organic phosphorus, respectively. As already mentioned, they gave an intense Schiff (Feulgen) test. A 0.125 gm. quantity dissolved in 7.5 ml. of hot ethanol and added dropwise with shaking to 50 ml. of 2,4-dinitrophenylhydrazine reagent (15) resulted in the immediate precipitation of the orange hydrazones which were filtered after standing 18 hr. at

TABLE II

THE BODY WALL PHOSPHOLIPIDS

	Preparation	
	1	2
Total phospholipids (% of total lipids)	38	39
Phosphoacetals (% of total phospholipids)		
Aldehydes	15.4	17.5
Acetals*	27	30
Acetal-free phospholipids		
Labile P (% of total P)	84	88
N : P (molar)	1.03	1.08
Choline : P (molar)	0.40	0.47
Ethanolamine	Abundant	Abundant
Serine	Trace	Trace
Fatty acids		
% of phospholipids	70	70
Iodine number	65	66

* *Calculated from the aldehyde as the C_{18} acetal of glycerylphosphorylethanolamine.*

room temperature. Yield, 0.116 gm. (56%, calculated as the C_{18} 2,4-dinitrophenylhydrazone). Recrystallization of this product from 85% ethanol gave 0.070 gm. of soft orange crystals, m.p. 79°–80°, which were probably a mixture of the hydrazones of several aldehydes. Fractionation of this mixture was not attempted.

In the reproductive tissues of ascaris the large quantities of unsaponifiable lipids consist mainly of ascaryl alcohol, although cholesterol and a saturated sterol are also present (5, 7). Examination of the unsaponifiables isolated from the body wall resulted in a similar division into ether-soluble and -insoluble components. For example, 0.324 gm. of unsaponifiables yielded 0.195 gm. (60%) of ether-insolubles, m.p. 76°–78° C. After two recrystallizations from chloroform – petroleum ether, a white, waxy substance was obtained which seemed to be identical with ascaryl alcohol prepared from reproductive tissues, as the melting point and mixed melting point were identical (83° C.). However, acetylation of this preparation in acetic anhydride – sodium acetate at 95° C., or in acetic anhydride – pyridine at 2° C. (14) gave a tough, film-like acetate, m.p. 58°–59° C., quite unlike the slightly waxy, granular diacetate, m.p. 50°–51° C., prepared from authentic ascaryl alcohol. This result was confirmed several times using other preparations of the ether-insoluble substance. It is probable, therefore, that the major unsaponifiable component of the body wall lipids is similar to, but not identical with, the ascaryl alcohol of reproductive tissues. The structure of either compound is not yet known.

Sterols were present in the ether-soluble fraction of the foregoing preparation of unsaponifiables. These were isolated as the digitonides (0.240 gm.), from which 0.051 gm. of free sterols (16% of the total unsaponifiables) were recovered. Their unsaturation (I.N., 57) and color factor (0.58) were similar to those of the reproductive tissue sterols, whose components were previously reported to be 75% unsaturated (cholesterol) and 25% saturated (5, 8).

TABLE III

LIPIDS OF THE MUSCLE* AND INTEGUMENT**

Fraction	Muscle		Integument	
	% of wet weight	Relative concentration	% of wet weight	Relative concentration
Total lipids	0.75	100	0.61	100
Phospholipids	0.37	49	0.25	41
Unsaponifiables	0.083	11	0.097	16
Triglycerides***	0.30	40	0.26	43

* *By analysis of 304 gm. of muscle (69.1 gm. dry weight).*
** *By analysis of 69 gm. of integument (18.0 gm. dry weight).*
*** *Triglycerides = total lipids − (phospholipids + unsaponifiables).*

Since for practical purposes the ascaris body wall is easily divided into two distinct parts, the muscle and the integument or cuticle, and as these tissues are radically different from one another in appearance and function, it was of interest to isolate and compare the lipids from each. The results (Table III) show a surprising similarity in the concentration of water, phospholipids, triglycerides, and unsaponifiables. Both tissues contained esterified volatile acids in the triglyceride fraction, and in each the main unsaponifiable component was the ascaryl alcohol-like substance already described. Sterols accounted for 12 and 17% of the total unsaponifiables in integument and muscle, respectively, as compared with 16% in the body wall as a whole.

Discussion

Flury (10) reported higher values for total body wall lipids than those determined repeatedly in this laboratory. It is probable that this discrepancy arises largely from the efficient washing technique (11) used in the present investigation, whereby non-lipid impurities are eliminated.

A more detailed analysis of the body wall lipids has provided several interesting results. In the phospholipid fraction (Table II), which is relatively large as is usual in muscle, acetals (as calculated from the isolated aldehydes) accounted for some 30% of the total phospholipids. The body wall phospholipids thus appear to be the richest known source of these poorly understood compounds, to which no specific function has yet been assigned. The remaining phospholipids appear to consist primarily of lecithins and cephalins, as judged by their lability (86%) in *N* sodium hydroxide at 37° C. and their high choline and ethanolamine content. Serine (phosphatidylserine) was found in traces only. The relatively small alkali-stable component of the acetal-free phospholipids may consist of spingolipids, for which, however, no direct analysis was made. The iodine number (65) of the phospholipid fatty acids was so low that a confirmatory examination was made of the acids prepared from body wall phospholipids isolated by the most careful procedures. These fatty acids appear to be the most highly saturated of any yet found in animal phospholipids. It would be interesting to find out whether this peculiarity of the phospholipid fatty acids is found in other animals living in a similarly warm (40° C.) environment.

Although the phospholipids contained no volatile acids, these were present in the triglycerides in about the same proportions as in the reproductive tissues (5), i.e. in amounts sufficient to account for about one-half the ester linkages with glycerol. As already shown (5) the triglyceride volatile acids are the same as those produced during the parasite's energy metabolism, probably from glycogen (3). The functional importance of the incorporation of such fermentation acids into triglycerides cannot be assessed at present. It is probable, however, that the muscle and integument triglycerides can be classed as structural lipids, since von Brand (1) was unable to detect any decrease in the lipid content of ascaris under conditions of severe starvation.

The presence of sterols in the adult body wall was not unexpected, as their presence was already established tentatively in another nematode, *Eustrongyloides ignotus* (2), and with certainty in the ascaris embryo (8) and adult female (7). More surprising was the presence as the main unsaponifiable substance of a higher alcohol, apparently closely related to ascaryl alcohol whose function in the vitelline membrane of the egg (8) is extracellular. It was pointed out, however, that the embryo itself appeared to contain small amounts of ascaryl alcohol, and von Brand and Winkeljohn (2) had previously isolated a similar substance from the male ascaris. These considerations, nevertheless, do not appear to provide a clue to the function of the alcohol in the muscle. Its function in the integument is more readily surmised, as this tissue provides a barrier between the parasite and its environment which is comparable in some ways to that provided by the vitelline membrane of the egg.

The nature and distribution of lipids in the integument, so similar to that occurring in the muscle, lends no support to the view that this protective "skin" is metabolically inert. Until more information is available, it would seem wise to regard the integument as a fully integrated living tissue, rather than as an inert, horny sheath.

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THE METABOLISM OF THE ERYTHROCYTE

IX. DIPHOSPHOPYRIDINE NUCLEOTIDASE OF ERYTHROCYTES[1]

By S. G. A. Alivisatos,[2] S. Kashket, and O. F. Denstedt

Abstract

The rabbit erythrocyte possesses an active DPN-ase which is firmly attached to the membrane of the cell. Evidently, the enzyme is oriented in the membrane in such a way as to be able to act upon DPN added to the external medium. The enzyme splits DPN at the bond linking the quaternary nitrogen of the nicotinamide moiety with the ribose component. Despite the release of an H^+ ion during hydrolysis of DPN, the activity of the enzyme remains practically constant over the pH range from 4.5 to 10.0. DPN-ase also splits nicotinamide from TPN, but its affinity for the latter is only about one third of that for DPN. Nicotinamide, adenine, and the substituted purine derivatives theobromine, theophylline, and xanthine inhibit DPN-ase, while compounds such as ribose, adenylic acid, caffeine, and nembutal are without effect in this respect. Of all the substances tested theobromine proved to be the most powerful inhibitor. The mode of enzyme–substrate attachment and the possible involvement of DPN-ase in ion transport are discussed in the light of these findings.

Rabbit erythrocytes afford an excellent source of DPN-ase* of the type which splits nicotinamide from DPN. In the erythrocyte the enzyme is associated exclusively with the cell membrane and apparently is confined, in its distribution, to the outer surface of the cell (1, 3). The properties of DPN-ase have been studied in our laboratory by means of the lactic, malic, and glucose-6-phosphate dehydrogenases present in the hemolyzate (1, 2). The present communication deals with a more direct study of the properties of the enzyme, the mode of combination with its substrate and the inhibition of its activity by various purine and pyrimidine compounds.

Experimental

Experimental Procedures

Manometric Experiments

In the manometric studies the method of McIlwain and Rodnight (21) was used with the conventional Warburg apparatus. The method entails the measurement of the volume of CO_2 evolved as the hydrolysis of DPN proceeds in the presence of bicarbonate buffer.

The volume of liquid in the Warburg flasks was kept at 2 ml. The medium contained NaCl in a final concentration of 0.127 *M*, and $NaHCO_3$, in final concentration of 0.025 *M*. DPN, when required, was used in concentration of the order of 3×10^{-3} *M*. In the majority of instances the enzyme (0.1–0.2 ml. of stroma-free hemolyzate) was placed in the side bulb of the vessel and

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* *The following abbreviations are used throughout this paper: DPN-ase, diphosphopyridine nucleotidase; DPN and TPN, di- and tri-phosphopyridine nucleotide, respectively; DPN.H, reduced diphosphopyridine nucleotide; SFH, stroma-free hemolyzate.*

the coenzyme (neutralized DPN) in the main compartment. The contents of the flasks were equilibrated with a gas mixture of 5% CO_2 and 95% N_2 at 37° C. The reaction then was initiated by tipping the contents of the side bulbs into the main compartment of the flasks and the evolution of CO_2 was followed at 5 or 10 min. intervals for 60 min. At the end of this period the contents of the vessels were deproteinized and the free nicotinamide determined by chemical analysis.

Estimation of nicotinamide.—Nicotinamide was estimated with the cyanogen-bromide reagent according to the procedure of McIlwain and Rodnight (21, 23). The initial removal of protein from the reaction mixture was accomplished by the use of $ZnSO_4$ and $Ba(OH)_2$ (27). The colorimetric readings were made with a Coleman Junior spectrophotometer at a wavelength of 420 mμ. The procedure permits the estimation of nicotinamide in the presence of DPN and is accurate within the concentration range of 0.05 to 0.5 μM.

Spectrophotometric Experiments

In the majority of our studies the spectrophotometric method of Kaplan *et al.* (14) was used for the estimation of DPN. The method is based on the reaction of DPN with cyanide (24, 5) with the formation of a complex which has an absorption maximum in the ultraviolet at 325 mμ. However, the quantity of cyanide added varied with the requirement of the individual experiments. A 1 *M* cyanide solution was added to the incubated mixtures to make a final volume of 7.0 ml., but in some cases, when the concentration of DPN was unusually high, the final volume was made up to 14.0 ml. The duration of the incubation period was usually 7.5 min., but in some experiments was 15 min. The volume of the incubated samples was kept at 0.6 ml. When particulate preparations were used the contents of the vessels were filtered after the addition of cyanide and before the spectrophotometric readings were made. In the majority of instances, however, filtration was not necessary as the stroma material readily dissolved on addition of the KCN (pH 11). Appropriate zero-time 'controls' accompanied each of the experimental vessels. The contents of the control vessels were the same as in the corresponding experimental flasks but, in the case of the former, the cyanide was added immediately after the addition of the enzyme. Both the experimental samples containing DPN and the zero-time controls were read against 'blanks' containing all the ingredients except DPN.

Estimation of Protein

Soluble proteins were usually determined by the spectrophotometric method of Warburg and Christian (31). A reading, taken at 340 mμ, indicated the degree of non-specific absorption and determined the applicability of the method. Insoluble stroma protein was digested with acid and estimated by the procedure of Johnson (13, 30). In a few cases, when the specimens were free from hemoglobin, the total protein was estimated by the biuret method as described by Gornall *et al.* (7).

Materials

Substrates

DPN was obtained from the Nutritional Biochemicals Corporation and from the Sigma Chemical Co.

DPN.H was prepared by reduction of DPN with hydrosulphite, according to the method described by Ohlmeyer (28). In later studies a commercial preparation (Sigma) was used which also had been prepared by reduction with hydrosulphite.

TPN was prepared according to the method of LePage and Mueller (16) and assayed by means of the glucose-6-phosphate dehydrogenase in the SFH from rabbit erythrocytes as described in a previous communication (2).

The coenzyme materials, which, in this study, were used as substrates, were assayed chemically either by the method of Potter, as described by LePage (15), or spectrophotometrically, as the cyano-derivatives, as described by Colowick *et al.* (5). The values for the molecular extinction coefficients used in the calculation of the results were those derived by Horecker and Kornberg for DPN.H (11) and by Colowick *et al.* (5) for the cyano-derivatives of the coenzyme, at 340 mμ.

Inhibitors

Nicotinamide, adenine, adenosine, adenosine-3′-phosphoric acid (yeast AMP), adenosine-5′-phosphoric acid (muscle AMP), ribose, xanthine, uracil, thymine, alloxan, benzimidazole, barbital, nembutal, caffeine, theobromine, theophylline, were commercial products.

Preparation of DPN-ase from Rabbit Erythrocyte

Rabbit red blood cells were washed with about five times their volume of 0.9% NaCl. The specimen was centrifuged at about 500 × *g* for 10 min. and the supernatant fluid was discarded. The washing procedure was repeated three or four times. Finally, the cells were suspended in sufficient saline (0.9% NaCl) to give a cell count of about 2.5×10^6 per mm^3. They were then hemolyzed by alternate freezing (in an acetone – dry-ice mixture) and thawing (at 37° C.), the operation being repeated three times. The stroma particles in the hemolyzate, being cohesive, are readily precipitable by centrifugation at low speed (1000 × *g*). The stroma material thus isolated is referred to in the text and the tables as "unwashed stroma".

In other experiments, the stroma residue was further washed from one ("once-washed stroma") to three times ("thrice-washed stroma"), each time with 50 times its volume of cold 0.9% NaCl (3° to 4°C.). This treatment removed a large portion of the soluble protein including hemoglobin, while the DPN-ase content remained unaltered. Thus, the specific activity of the preparation tended to increase. The specific activity of the thrice-washed stroma was about five times that of the unwashed preparation. The washings were found to be free of DPN-ase activity (cf. Table I). The stroma material from the unwashed or the thrice-washed preparation may be kept in the frozen state for several weeks without undergoing an appreciable loss of activity.

An effort was made to bring the enzyme into solution by freeing it from the other components of the membrane. The thrice-washed stroma, suspended in distilled water or phosphate buffer, was homogenized for 60 min. at 2° C. with a Potter–Elvehjem homogenizer. In these experiments numerous phosphate buffers, ranging in concentration from 0.01 to 0.1 *M*, and in pH from 4.9 to 9, were used. The homogenate in each case was centrifuged at 16,000 × *g* for 15 min. at 2° C. The supernatant extract, which often was slightly yellowish in color, was tested for activity and, in all cases, was found to be inactive. The precipitated stroma, on the other hand, retained all the activity of the original preparation. Hence the DPN-ase from rabbit erythrocytes, like that from brain (20) and spleen (32), is firmly attached to the tissue membranes. It differs, however, from the DPN-ase of *Neurospora crassa* in that the latter is readily extractable in soluble form with phosphate buffer (14).

Partial success in liberating the enzyme in soluble form was achieved by extracting an "acetone-dried" powder prepared from thrice-washed stroma, according to the method of Green, Needham, and Dewan (8). The data in Table I indicate that the acetone-dried material contained the DPN-ase in a fairly active form. Although somewhat less active than the original stroma, the acetone-dried powder is much more stable and, when stored in the cold, or in perfectly dry atmosphere, retains its activity for many months. On extraction of the powder with distilled water for one to three hours at 2° C. in a Potter–Elvehjem homogenizer, and centrifugation of the sample at 24,000 × *g*, a slightly yellowish colored supernatant was obtained which contained from 0.5 to 1% of the DPN-ase activity of the original acetone-dried powder suspension (cf. Table I).

Since this paper was written, one of the authors (S.K.) has applied successfully, the *n*-butanol method of Morton (25) and has obtained a soluble preparation of DPN-ase from the stroma material. However, the yields of the enzyme were quite low.

Results

1. Cytological Distribution of DPN-ase in the Rabbit Erythrocyte

Table I indicates the specific activity of the DPN-ase from the various fractions of rabbit blood. The specific activity of whole blood was essentially the same as that of the suspension of washed erythrocytes. Both the plasma and SFH were devoid of activity while the stroma contained all the enzyme of the erythrocyte.

In another series of experiments (Table II) the relative DPN-ase activity of whole blood and of the hemolyzate prepared by freezing and thawing whole blood were compared. Similarly, a comparison was made of activities of intact red cells and of hemolyzed, washed (plasma-free) cells. In both cases it was found that hemolysis did not alter the activity of the enzyme preparation.

It is evident that the DPN-ase is confined to the red cell membrane and that the enzyme of the intact cell is so distributed that it can split DPN added to the external medium.

TABLE I

SPECIFIC ACTIVITY* OF THE DPN-ase IN VARIOUS FRACTIONS OF RABBIT BLOOD

Preparation	Protein (mgm./ml.)	Dry residue (mgm./ml.)	Units† (per ml.)	Specific activity*	
				Per mgm. protein	Per mgm. dry residue
Blood‡	320.0	177.2	740	0.19	0.32
Suspension§ of red cells	129.0	69.4	294	0.18	0.34
Unwashed stroma	130.0	81.4	2025	1.25	2.00
Once-washed stroma	35.6	35.7	1845	4.16	4.16
Twice-washed stroma	26.8	31.7	1965	5.89	4.95
Thrice-washed stroma	28.4	29.7	2360	6.67	6.35
Acetone-dried powder	7.0	10.0	510	5.85	5.00
Extract of acetone-dried powder	0.9	—	54	4.85	—
Stroma-free hemolyzate	123.8	69.0	0	—	—
Fluid from first washing	10.8	13.8	0	—	—
Fluid from second washing	1.3	1.0	0	—	—
Fluid from third washing	0.3	0.7	0	—	—
Plasma	—	3.9	0	—	—

* *Expressed as μM. of DPN split per mgm. of protein (or dry residue) per hour.*

† *One unit = the amount of enzyme which will split 1 μM. of DPN per hour under these conditions.*

‡ *Cell count = 5.4 × 10⁶ cells/mm³.*

§ *Cell count = 1.8 × 10⁶ cells/mm³.*

DPN determined according to Kaplan et al. (14). Initial concentration of DPN = 1.37 × 10⁻³ M.

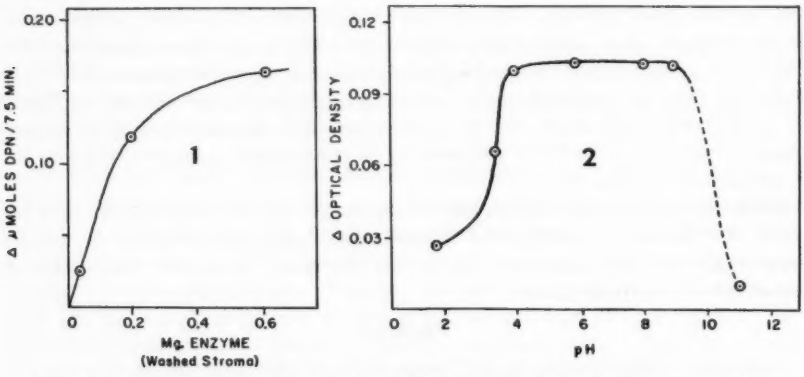

FIG. 1. Influence of the concentration of DPN-ase on the rate of hydrolysis of DPN. Concentration of DPN: 9.5×10^{-4} *M*.

FIG. 2. Influence of pH on the activity of DPN-ase. Concentration of DPN: 1.6×10^{-4} *M*.

TABLE II

EFFECT OF HEMOLYSIS ON DPN-ase ACTIVITY

Enzyme preparation	Dry residue (mgm./ml.)	Change in optical density at 340 mμ
Whole blood	8.76	0.158
Hemolyzed blood	8.76	0.165
Washed erythrocytes	4.92	0.120
Hemolyzed washed erythrocytes	4.92	0.116

2. Liberation of Nicotinamide by DPN-ase

The glycosidase character of the erythrocyte DPN-ase is readily demonstrable. Table III indicates the equivalence of carbon dioxide liberated manometrically and the nicotinamide released upon incubation of DPN in the presence of stroma. In all cases the ratio of the number of μM. of CO_2, liberated from the bicarbonate buffer, to the number of μM. of nicotinamide released was close to unity. Furthermore, Colowick *et al.* (5) have shown that the reaction between DPN and cyanide is dependent upon the presence of the pyridinium–ribose bond. The failure of the reaction to occur when DPN is exposed to the various erythrocyte preparations provides further proof that the DPN-ase splits DPN at the bond between nicotinamide and ribose.

3. Influence of Hydrogen-ion Concentration on the Activity of DPN-ase

The action of the DPN-ase from rabbit erythrocytes was measured over a range of pH values between 1.25 and 10.9 using a phosphate buffer concentration of 0.067 *M*. The enzyme preparation was an acetone-dried stroma preparation representing a concentration of 0.3 mgm. of protein per ml. The reaction was carried out at 37° C. and was followed for a period of 7.5 min.

As indicated in Fig. 2 the maximum activity was obtained over the pH range from 4.5 to 10.2. Above the latter limit the activity falls off abruptly and above pH 10.9 the enzyme becomes irreversibly inactivated.

Control experiments with DPN alone (1.55×10^{-3} *M*) were carried out to determine its stability between pH 1.25 and 10.9 at 37° C. The nucleotide was found to be stable only between pH 1.25 and 9.1 during the experimental period of 15 min. Consequently in the experiments at pH values above 9.1, appropriate correction was necessary to take into account the non-enzymatic hydrolysis of the DPN.

4. Comparative Action of DPN-ase on DPN and TPN

For these experiments an acetone-dried powder prepared from well-washed stroma from rabbit erythrocytes was used as the enzyme preparation in a concentration of 0.6 mgm. per ml. The substrates were used over a range of

TABLE III

EVOLUTION OF CO_2 AND THE RELEASE OF NICOTINAMIDE FROM DPN

Enzyme preparation	Dry residue (mgm./ml.)	CO_2* (μM./mgm./hr.)	Nicotinamide* (μM./mgm./hr.)	Molar ratio CO_2/nicotinamide
Unwashed stroma	1.91	2.22	2.26	0.98
Twice-washed stroma	1.09	3.60	3.66	0.98
Thrice-washed stroma	1.00	3.84	3.84	1.00

* *CO_2 evolution determined manometrically; the values have been corrected for CO_2 retention according to the procedure of Zatman et al. (32); nicotinamide determined by cyanogen-bromide method as described by McIlwain and Rodnight (21).*

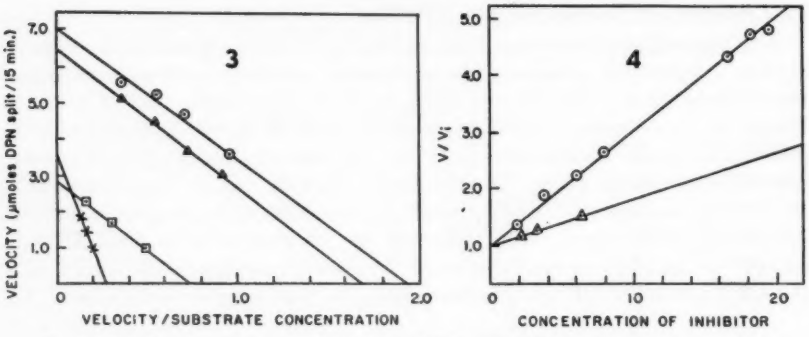

FIG. 3. Michaelis constants obtained for DPN and TPN in the presence of various buffers.

○ K_{DPN} in pyrophosphate buffer, pH 6.5: $3.9\times10^{-4}M$.
△ K_{DPN} in propionate buffer, pH 4.9: $4.8\times10^{-4}M$.
□ K_{DPN} in phosphate buffer, pH 4.9: $3.9\times10^{-4}M$.
× $K_{TPN} = 1\times10^{-3}M$.

FIG. 4. Inhibition of DPN-ase by adenine and nicotinamide.
△ Adenine: Moles$\times10^{-4}$/liter.
○ Nicotinamide: Moles$\times10^{-3}$/liter.

concentrations from 0 to 1.7×10^{-3} *M* in the case of DPN, and from 0 to 13.55×10^{-3} *M* for TPN. The reaction media were buffered with sodium phosphate (0.033 *M*) at pH 4.9 and the runs were made at 37° C. during a period of 15 min.

A modified Lineweaver-Burk plot (17, 10) of the results is presented in Fig. 3. In this presentation the slope of the curves is equal to the K_m.

The Michaelis constants K_{DPN} and K_{TPN} were found to be of the order 4×10^{-4} *M* and 1×10^{-3} *M*, respectively. Thus the affinity of DPN-ase for DPN is about 2.5 times as great as that for TPN.

5. *Resistance of DPN.H to the Action of DPN-ase*

In a series of experiments the action of DPN-ase on reduced DPN was examined by the spectrophotometric method of Kaplan *et al.* (14) (see p. 46). DPN.H (1.4×10^{-3} *M*) (Sigma Chemical Co.) in one vessel, and DPN (1.4×10^{-3} *M*) in another, were incubated with DPN-ase (acetone-dried powder of stroma, 7 mgm. protein/ml.) for 7.5 min. at 37° C. At the end of this period 6.5 ml. of 1 *M* cyanide were added to the latter vessel and the same quantity of water, to the former. The optical density was determined at 340 mμ in the spectrophotometer against appropriate controls.

As indicated in Table IV the DPN.H in flask No. 1 remained intact while 0.51 μM. of DPN in No. 2 had undergone hydrolysis.

6. *Inhibition of DPN-ase by the Components of DPN*

This series of experiments was carried out to test the effects of each of the components of the DPN molecule on the action of DPN-ase towards DPN. The conditions of typical experiments are given in Table V.

TABLE IV

SPECIFICITY OF THE DPN-ase IN THE RABBIT ERYTHROCYTE

	Substrate	Quantity of substrate utilized (μM.)
1.	DPN.H	0.008
2.	DPN	0.510

TABLE V

INFLUENCE OF COMPONENTS OF DPN ON THE ACTIVITY OF DPN-ase

Enzyme concentration (mgm. protein/ml.)	DPN (molarity)	Component listed as inhibitor	Buffer	pH	K values
6.0	5.4×10^{-4}	Nicotinamide 0 to $2 \times 10^{-2}M$	Phosphate 0.033M	4.9	K_i $2.0 \times 10^{-3}M$
6.0	1.4×10^{-4}	Nicotinamide 0 to $1.6 \times 10^{-2}M$	"	"	$3.8 \times 10^{-3}M$
1.17	1.5×10^{-3}	Adenine 0 to $3.3 \times 10^{-4}M$	"	"	$2.4 \times 10^{-3}M$
1.17	1.6×10^{-3}	Ribose 0 to $5 \times 10^{-1}M$	"	"	$6.5 \times 10^{-1}M$
0.58	7.6×10^{-4}	Adenosine 0 to $1 \times 10^{-2}M$	"	"	$1 \times 10^{-2}M$
0.58	7.2×10^{-4}	Adenylic acid (muscle) 0 to $3.5 \times 10^{-1}M$	"	"	$1.8 \times 10^{-1}M$
0.58	7.2×10^{-4}	Adenylic acid (yeast) 0 to $1.0 \times 10^{-1}M$	"	"	$1.1 \times 10^{-1}M$
0.58	0 to 1.5×10^{-3}	None	Pyrophosphate 0.033M	6.5	K_{DPN} $3.9 \times 10^{-4}M$
0.58	0 to 1.4×10^{-3}	None	Propionate 0.033M	4.9	$4.8 \times 10^{-4}M$
0.58	0 to 1.7×10^{-3}	None	Phosphate 0.033M	4.9	$4.9 \times 10^{-4}M$

The experiments were carried out at 37.5° C. over a period of 15 min.

Nicotinamide

Nicotinamide is a potent inhibitor of the DPN-ase of the rabbit erythrocyte. Fig. 4 indicates a modified Lineweaver-Burk plot (10) of the data from a typical experiment.

The plot of results indicates that the inhibition is of the competitive type with the K_i equal to 2.0 to 3.8×10^{-3} M (see Table V). It should be emphasized that our presentation of the nature of the nicotinamide inhibition is strictly an interpretation of the graphical analysis of our data. These data were collected from experiments in which the concentration of DPN was kept constant and the concentration of nicotinamide was varied. If the concentration of nicotinamide be held constant and the concentration of DPN varied, the values obtained are not consistent with a competitive type of inhibition. This is true especially with concentrations of DPN higher than

2.0×10^{-3} *M*. However, such discrepancies are not so unexpected and may be due to (*a*) the increase in the concentration of free nicotinamide due to cleavage of DPN, and (*b*) a two-point attachment of DPN on the enzyme surface (see p. 58).

Adenine

This component of DPN, by virtue of its terminal position in the molecule, was assumed to be a possible inhibitor of DPN-ase. Because of the low solubility of the purine it was possible to work only with low concentrations. The results are indicated in Fig. 4. It is evident that adenine is a strong inhibitor of the enzyme and that the inhibition is of the competitive type (see Table V for K_i values).

Nicotinamide and Adenine Together

Nicotinamide and adenine, when used together with a washed-stroma preparation, produced an additive inhibition of DPN-ase. The data from typical experiments are given in Table VI. In many cases the inhibition produced by the two compounds together was less than the sum of the inhibitions of the compounds when used separately. With certain concentrations, however, the inhibition reached a maximum at about 90% of complete inhibition of the enzyme.

D-Ribose

This component of DPN also inhibits DPN-ase, but, as indicated in Fig. 5, the inhibition is of small magnitude compared with that of nicotinamide or adenine (K_i see Table V).

Adenosine

This nucleoside, being sparingly soluble in water, could be used only in low concentrations. It appears to have a slight inhibitory effect on DPN-ase (K_i see Table V).

TABLE VI

COMPARISON OF THE INHIBITORY ACTION OF NICOTINAMIDE AND ADENINE

Experiment	Nicotinamide ($\times 10^{-3} M$)	Adenine ($\times 10^{-3} M$)	Per cent inhibition
1*a*	4.8	—	30
b	4.8	4.25	40
c	4.8	8.50	47
d	4.8	12.75	57
e	—	8.50	26
2*a*	—	8.50	29
b	4.0	8.50	42
c	12.0	8.50	60
d	24.0	8.50	76
e	48.0	8.50	87
f	12.0	—	51

Initial DPN concentration = 8×10^{-3} *M*.

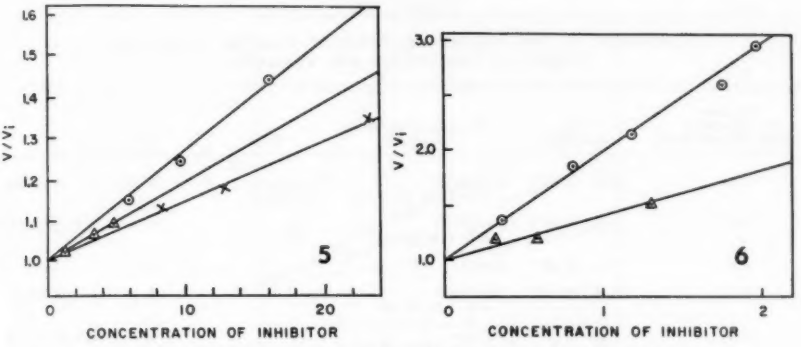

FIG. 5. Inhibition of DPN-ase by muscle and yeast adenylic acid and by ribose.
× Muscle adenylic acid: Moles×10^{-2}/liter.
○ Yeast adenylic acid: Moles×10^{-2}/liter.
△ Ribose: Moles×10^{-1}/liter.

FIG. 6. Inhibition of DPN-ase by xanthine and thymine.
△ Xanthine: Moles×10^{-2}/liter.
○ Thymine: Moles×10^{-2}/liter.

Adenylic Acid from Muscle and Yeast

These nucleotides, being readily soluble in water, could be tested over a wide range of concentrations. As indicated in Fig. 5, both compounds cause a relatively weak inhibition of DPN-ase, apparently of the competitive type. The degree of inhibition was comparable with that of ribose. (K_i values see Table V.)

Phosphate and Pyrophosphate

To ascertain whether the phosphate ions in the phosphate buffer, as used in the afore-mentioned experiments, exert an inhibitory action on DPN-ase, experiments were repeated using propionate and pyrophosphate buffers instead of phosphate buffer. The parallel curves in Fig. 3 indicate that neither phosphate nor pyrophosphate inhibits the enzyme. (K_i values see Table V.)

7. *The Influence of Purine and Pyrimidine Derivatives and Analogues on the Activity of DPN-ase*

Xanthine

Xanthine was dissolved in a slight excess of 0.2 *N* NaOH and the solution was neutralized carefully with 0.2 *N* HCl until the yellow coloration was discharged. The pH of the solution was then 8.65.

These solutions manifest a considerable absorption in the ultraviolet at 340 mμ and the absorption follows Beer's law within the range of concentrations, 0.33 to 1.34 × 10^{-2} *M*. In the estimation of DPN, therefore, appropriate correction was made for the absorption due to xanthine.

The results as presented in Fig. 6 indicate that xanthine inhibits DPN-ase in a competitive manner and in degree comparable with that of adenine (K_i see Table VII).

TABLE VII

COMPARISON OF THE INHIBITORY ACTION OF VARIOUS PURINE AND PYRIMIDINE DERIVATIVES AND ANALOGUES

Enzyme concentration (mgm. protein/liter)	DPN (molarity)	Compound tested as inhibitor	Buffer	pH	K_i
0.5	7.9×10^{-4}	Xanthine 0 to $1.23 \times 10^{-2}M$	Phosphate 0.033*M*	8.58	$7 \times 10^{-3}M$
0.5	7.9×10^{-4}	Thymine 0 to $3 \times 10^{-2}M$	"	4.9	$2.8 \times 10^{-3}M$
0.5	7.9×10^{-4}	Uracil	"	4.9	—
0.5	7.9×10^{-4}	Nembutal 0 to $7 \times 10^{-3}M$	"	8.2	$1 \times 10^{-3}M$
0.5	7.75×10^{-4}	Caffeine 0 to $4.48 \times 10^{-1}M$	"	4.9	$1.2 \times 10^{-1}M$
0.5	7.75×10^{-4}	Theophylline 0 to $3.67 \times 10^{-2}M$	"	4.9	$2.5 \times 10^{-3}M$
0.5	7.75×10^{-4}	Theobromine 0 to $3.67 \times 10^{-2}M$	"	4.9	$6.6 \times 10^{-4}M$
0.5	7.75×10^{-4}	Benzimidazole 0 to $7.6 \times 10^{-2}M$	"	4.9	$2.5 \times 10^{-3}M$

The experiments were carried out at 37.5° C. over a period of 15 min.

Thymine

The inhibition with this pyrimidine is of the competitive type and comparable in degree with that of adenine (see Fig. 6 and Table VII).

Uracil

Owing to the extremely low solubility of uracil in water only a very weak concentration of the pyrimidine could be obtained even in a saturated solution at pH 4.9 and at 37.5° C. Uracil also appears to inhibit DPN-ase (see Table VII).

Nembutal, Barbital, and Alloxan

Nembutal exerts a weak inhibition of DPN-ase while barbital and alloxan are without effect (see Table VII).

Theobromine, Theophylline, and Caffeine

Of the three methylated purine derivatives theobromine was found to be by far the most potent inhibitor of DPN-ase; indeed it is the strongest inhibitor of all the compounds tested in our studies, the K_i being of the order of 6.6×10^{-4}. As indicated in Fig. 7, theophylline is comparable with nicotinamide in inhibitory activity. (K_i values see Table VII).

Benzimidazole

This analogue of purine was tested over a range of concentrations from 0 to 7.6×10^{-2} *M*. Its inhibitory action on DPN-ase, as indicated in Fig. 7 and Table VII, is comparable with that of nicotinamide.

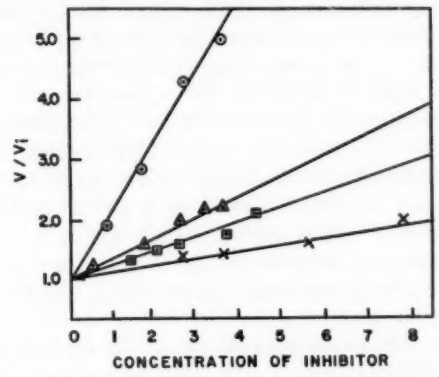

FIG. 7. Inhibition of DPN-ase by theobromine, theophylline, caffeine, and benzimidazole.

Concentrations of inhibitors: △ Theobromine, moles × 10^{-3}/liter; ○ theophylline, moles × 10^{-3}/liter; □ caffeine, moles × 10^{-1}/liter; × benzimidazole, moles × 10^{-3}/liter.

Discussion

The findings in the present study confirm our earlier observations (1, 3) that the DPN-ase of the rabbit erythrocyte is firmly attached to the cell membrane or stroma. The enzyme evidently is distributed in the membrane in such a manner that it is capable of reacting with substrate in the external medium since the intact rabbit erythrocyte can destroy exogenous DPN. As a corollary of this observation it is evident that DPN cannot exist in the circulating blood plasma.

The above-mentioned observation has an implication also for the distribution of DPN-ase in other somatic cells. It is well known that the DPN-ase of the glycosidase type is associated with the cell residues and that maceration of tissues leads to the rapid destruction of the endogenous DPN (6, 22, 21, 26). In the light of our findings in the rabbit erythrocyte these observations suggest that the DPN-ase may be present also in the membrane of all somatic cells. In some tissues, however, it may not be confined exclusively to the membrane since Sung and Williams (29) have observed that the DPN-ase of rat liver is contained in the nuclei, microsomes, and the supernatant fluid, but is absent from the mitochondria. Since the nuclear fractions may have been contaminated with membranous residues, it is possible that the enzyme is contained also in the membrane of the liver cells.

The hydrolysis of DPN by DPN-ase is attended by the release of a positive charge in the form of a proton and the conversion of a quaternary to a trivalent nitrogen. In DPN the positive charge is associated with the negative charges on two of the hydroxyls of the pyrophosphate group both of which are dissociated at pH 5 and higher. It is of interest that this proton-yielding reaction, as catalyzed by the DPN-ase of the rabbit erythrocyte, is independent of the H^+ concentration of the medium within the pH range of 4.5 to

10. The peculiar distribution of DPN-ase, at least in the rabbit erythrocyte, and the potential proton-liberating capacity of the enzyme suggest that DPN-ase may be involved in the transport of ions across the cell membrane of the rabbit erythrocyte. One feature against such an hypothesis is the fact that during hydrolysis of a mole of DPN about 8200 calories are liberated and presumably dissipated as heat (32). However, this energy may be used; indeed, Alivisatos and Woolley (4) recently have succeeded in utilizing this energy for the synthesis of a pyrophosphate of 4-amino-5-imidazole-carboxamide riboside and of adenylic acid.

DPN-ase is capable also of splitting nicotinamide from TPN but its affinity for the latter is only about one third that for DPN. McIlwain (19) observed that the DPN-ase of brain does not hydrolyze reduced DPN. We have observed similarly that the enzyme of the rabbit erythrocyte does not split the reduced form, DPN.H.

The inhibitory action of nicotinamide on DPN-ase, first observed by Mann and Quastel (22), has been demonstrated with many tissues and organisms. Even the enzyme from *Neurospora*, which according to earlier reports (14) was supposed to be unaffected by nicotinamide, has been shown more recently to be inhibitable with higher concentrations of the amide (32). Numerous workers (9, 22, 21) have demonstrated that DPN-ase in various animal tissues is about equally sensitive to nicotinamide inhibition. The inhibition apparently is not strictly competitive in character (32). These observations apply equally well to the DPN-ase of the rabbit erythrocyte.

In our study of the inhibition of DPN-ase of the rabbit erythrocyte by various substances adenine proved to be as potent an inhibitor as nicotinamide. In contrast with the action of these terminal units of the DPN molecule the intermediate components of the molecule had little or no inhibitory action. Thus ribose produced only slight interference when present in about 100 times the effective concentration of nicotinamide or adenine. Inorganic phosphate or pyrophosphate caused no inhibition. Adenosine and adenylic acid, like ribose, were only about 1/100th as active as nicotinamide or adenine. The powerful inhibitory activity of these last mentioned compounds and the circumstance that they occupy the terminal positions in the DPN molecule suggest that, in the normal action of DPN-ase, DPN, through its terminal units, may assume a two-point attachment to the enzyme. When free nicotinamide and adenine are added to the incubation medium containing DPN-ase the inhibitory activity of the two substances in low concentration is summated. At higher concentrations, however, the combined activities are no longer additive. The latter result is to be expected considering that the inhibition of the enzyme can be produced by nicotinamide or by adenine separately and that with a purely random uptake of the inhibitors by the specific sites on the enzyme a relatively greater effect per mole of inhibitor is to be expected when the inhibitors are present in the lower concentrations.

It is conceivable, furthermore, that the rate of hydrolysis of DPN by DPN-ase may be inhibited or retarded in the presence of an excessive concen-

tration of the DPN. Under these conditions many of the DPN molecules may assume only a one-point attachment and thus not only become unamenable to hydrolysis, but prevent other substrate molecules from assuming the favorable two-point combination. This is difficult to confirm experimentally because of the rapid liberation of nicotinamide during hydrolysis, and the inhibitory effect on the enzyme.

Of all the compounds related to adenine that were tested in our study theobromine proved to be the most powerful inhibitor of DPN-ase*. Next in order were theophylline and xanthine, these being comparable with nicotinamide in their inhibitory action. Caffeine produced inhibition only when present in relatively high concentration and thus is comparable with ribose in activity.

The strong inhibition by the dimethylxanthines is of considerable interest since an effective concentration could appear in the circulation after administration of a therapeutic dose of 0.3 gm. of theobromine or theophylline. With such a dose the concentration in the body fluids of a 70 kgm. individual would be several hundred times that of DPN normally present in the tissues. Such a concentration, or even 1/100th as much, is sufficient to cause complete inhibition of DPN-ase activity *in vitro*. Whether the inhibitory action on DPN-ase has any connection with the powerful pharmacological effects of theobromine and theophylline is not known.

Of the pyrimidine derivatives tested thymine proved to be comparable with nicotinamide in inhibitory activity. Uracil, because of its low solubility in water, gave indefinite results. Alloxan and the barbiturates had no inhibitory effect on the enzyme.

Benzimidazole, a purine analogue, was found to be comparable with thymine and nicotinamide in inhibitory activity. Hosein (12) in our laboratory observed that benzimidazole inhibits the activity of a large number of enzyme systems including the DPN- and TPN-linked dehydrogenases†.

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** Phenosafranine is reported to have an even stronger inhibitory action on the DPN-ase of nerve tissue (18). From values given by McIlwain (18), and assuming that the inhibition by phenosafranine is of the competitive type, the K_i would be approximately $3 \times 10^{-5} M$. In the case of nerve tissue, therefore, the affinity of the inhibitor for the enzyme is stronger than that of the substrate. However, in view of the difference in inhibitory activity of a given substance on the DPN-ase from various sources (33) it is difficult to predict whether phenosafranine will inhibit the DPN-ase of the rabbit erythrocyte.*

† In the light of recent observations by Alivisatos and Woolley (4) it is possible that benzimidazole undergoes combination with carbon-1 of the ribose component of DPN in much the same manner as does 4-amino-5-imidazolecarboxamide.

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STUDIES ON THE PRESERVATION OF BLOOD

IV. THE INFLUENCE OF ADENOSINE ON THE GLYCOLYTIC ACTIVITY OF THE ERYTHROCYTE DURING STORAGE AT 4° C.[1]

By David Rubinstein, Shelby Kashket, and Orville F. Denstedt

With the technical assistance of Sheila M. Gosselin

Abstract

Human blood was stored in citrate–dextrose and acidified citrate–dextrose media with and without the addition of adenosine. In both preservative media the adenosine induced an increase in the esterification of phosphate mainly in the form of 2,3-diphosphoglycerate, and also an increased production of lactate. The increase in lactate formation could not be accounted for entirely by the amount of glucose utilized and it was apparent that the extra amount of lactate must have been derived from the utilization of the ribose component of adenosine. The pH of the blood samples containing adenosine was found to be higher than that of the corresponding control samples. It has been demonstrated that adenosine added to blood is first converted to inosine by deamination and the latter, in turn, is converted to hypoxanthine. The probable route by which the ribose moiety of adenosine is metabolized in the erythrocyte is discussed.

Among the prominent changes that occur in the erythrocyte during storage is the progressive hydrolysis of the organic phosphate esters of the glycolytic system with liberation of inorganic phosphate. In blood, mixed with a neutral isotonic citrate–dextrose medium and kept at 4° C., the hydrolysis of these esters is delayed, or occurs very slowly, until the 12–15th day of storage. Thereafter, it proceeds at a relatively rapid rate. In the acidified citrate–dextrose medium, on the other hand, the hydrolysis of many of the organic phosphates in the cells proceeds from the beginning of storage (3).

Gabrio and Huennekens (10) have observed that adenosine, added to preserved blood specimens, induces a resynthesis of the phosphate esters in the red cells. The ability of these cells to survive in the circulation after transfusion also is improved (9). The present authors have studied the mechanism by which adenosine produces these changes and have compared the metabolic behavior of erythrocytes during storage with or without added adenosine in neutral and acidified preservative media.

Methods

Blood from a normal donor was collected into the two types of 'preservative' medium, namely: (*a*) a neutral isotonic mixture of sodium citrate and dextrose, designated in this paper as 'CD', and (*b*) acidified citrate–dextrose, commonly referred to as 'ACD'. The proportions of blood to preservative were as follows:

(*a*) Five parts of blood to 1.5 parts of CD (containing 2.1% trisodium citrate dihydrate and 1.7% anhydrous dextrose).

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Contribution from the Department of Biochemistry, McGill University, Montreal, Quebec. This work was supported by the Defence Research Board of Canada, Grant number 9350-01, Project D 50—93050-01.

(*b*) Four parts of blood to one part of ACD (containing 1.37% of trisodium citrate, 0.5% of citric acid monohydrate, and 2.5% of dextrose).

For storage experiments with added adenosine, the latter was added to the preservative medium before it was autoclaved. The quantity added was such as to give a concentration of 0.4% in the final blood sample.

All the blood specimens were stored at 4° C. and were agitated gently for a few minutes each day to resuspend the cells. At intervals of three days aliquots of the stored specimens were withdrawn, with aseptic precautions, for the estimation of glucose (13), phosphate (6), pyruvic acid (8), and lactic acid (1), using procedures indicated by these references. In addition, a fractional analysis of various organic phosphate esters was carried out according to a procedure previously outlined (14). For convenience of discussion these fractions will be designated by the following symbols:

P_i — Inorganic phosphate.

P_L — 'Labile' organic phosphate esters which are hydrolyzable within seven minutes in *N* HCl at 100° C. This fraction represents the two terminal phosphate groups of ATP and the phosphate attached to carbon-1 of hexose or pentose.

P_M — Organic esters of moderate stability which undergo hydrolysis within the interval between the 7th and 100th min. in *N* HCl at 100° C. These esters include the phosphoric esters of the hydroxyl on the terminal carbon atom of hexose and pentose sugars.

P_S — Stable phosphate esters which are not hydrolyzable by treatment of the red cells in *N* HCl at 100° C. up to 100 min. This fraction consists mainly of the phosphate groups of 2,3-diphosphoglycerate (DPG).

Results

The curves in Fig. 1 indicate the progress of the enzymatic hydrolysis of the 'stable' phosphate esters (P_S) and the accompanying increase in inorganic phosphate (P_i) of the erythrocyte during storage of blood in ACD and CD at 4° C. In the CD sample the liberation of P_i from the esters by hydrolysis is delayed until the 10th or 12th day while, in ACD, it increases from the onset of storage. In both specimens the enzymatic hydrolysis of DPG accounted for about 75% of the increase in the P_i fraction during the 32-day period of storage. In the samples to which adenosine had been added, on the contrary, the P_i was almost completely esterified and the content of 2,3-DPG was increased.

The curves in Fig. 2 indicate the corresponding behavior of the P_M fraction in the specimens preserved in CD and ACD. In the samples containing added adenosine this fraction was progressively increased during the storage period whereas in those without adenosine it was decreased. In the fraction containing adenosine a pronounced rise in the P_M occurred and was followed in some of the samples (e.g. in the ACD specimen referred to in Fig. 2) by a progressive decrease.

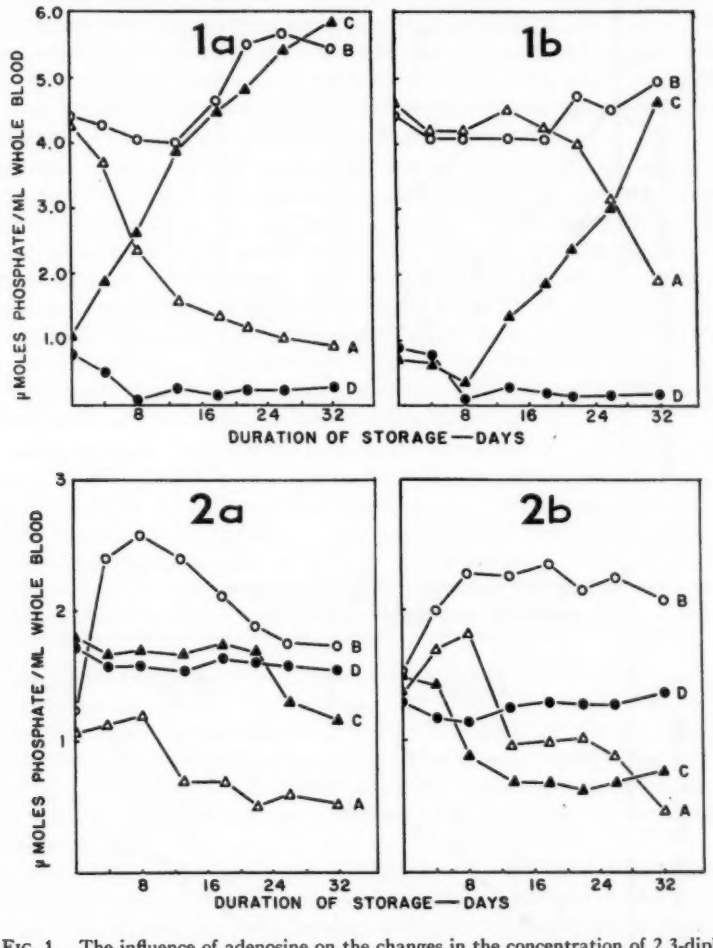

FIG. 1. The influence of adenosine on the changes in the concentration of 2,3-diphosphoglycerate and inorganic phosphate in blood during storage in ACD (Fig. 1*a*) and CD (Fig. 1*b*).

Curve A. — Fraction P_g (DPG), without adenosine.
Curve B. — Fraction P_g (DPG), with adenosine.
Curve C. — Fraction P_i, without adenosine.
Curve D. — Fraction P_i, with adenosine.

FIG. 2. Influence of adenosine on the changes in the concentration of adenosine triphosphate and hexosephosphates in erythrocytes during storage at 4° C. in ACD (Fig. 2*a*) and CD (Fig. 2*b*).

Curve A. — Hexosephosphates, without adenosine.
Curve B. — Hexosephosphates, with adenosine.
Curve C. — ATP, without adenosine.
Curve D. — ATP, with adenosine.

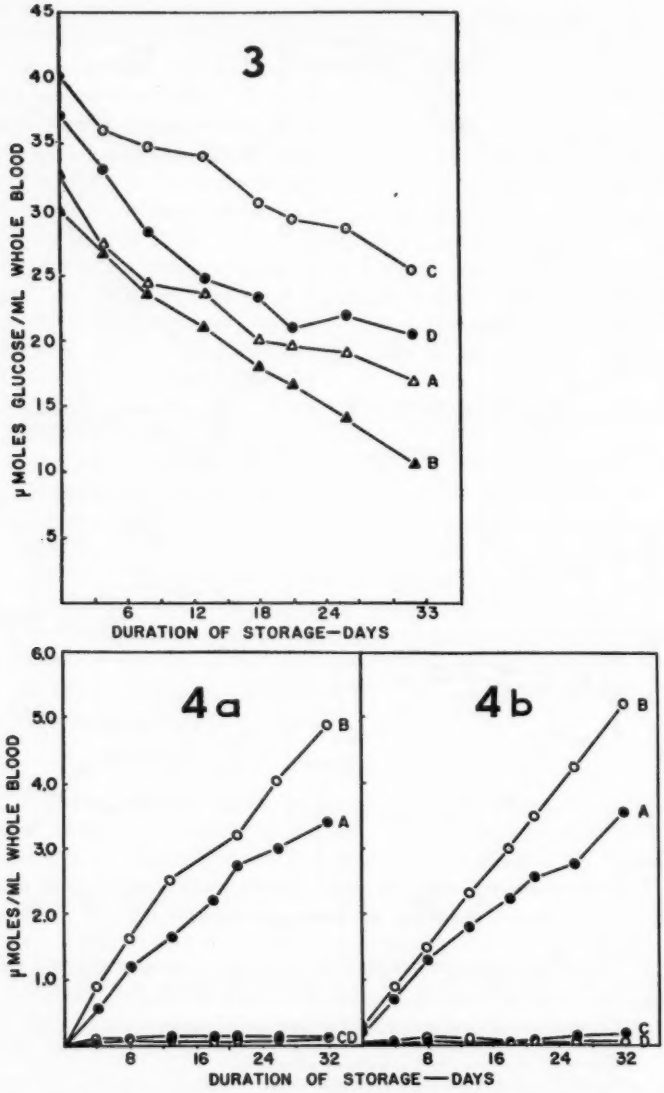

FIG. 3. The influence of adenosine on the utilization of glucose in blood preserved in CD and ACD at 4° C.

Curve A. — In CD without adenosine. Curve C. — In ACD without adenosine.
Curve B. — In CD with adenosine. Curve D. — In ACD with adenosine.

FIG. 4. The influence of adenosine on the accumulation of lactate and pyruvate in blood preserved in ACD (Fig. 4*a*) and CD (Fig. 4*b*).

Curve A. — Lactate, without adenosine. Curve C. — Pyruvate, without adenosine.
Curve B. — Lactate, with adenosine. Curve D. — Pyruvate, with adenosine.

It is well known that, despite the earlier breakdown of the majority of the phosphate esters in the erythrocyte during storage in ACD, ATP is relatively slowly hydrolyzed in blood at the lower pH (7.0), whereas at the more normal pH (7.4) of the CD preservative, it is hydrolyzed more rapidly. It is evident from Fig. 2 that the level of the P_L fraction (ATP) was considerably better maintained in the specimens containing adenosine.

The rate of utilization of glucose by the erythrocyte during storage, as indicated in Fig. 3, was only slightly (*ca.* 20%) stimulated by the presence of adenosine in the CD or the ACD preservative. It is noteworthy in Fig. 4, on the other hand, that the rate of formation of lactate, which may be expected to reflect the rate of utilization of glucose, was accelerated by about 40% in the specimens containing adenosine. Only in the blood specimen preserved in CD without adenosine did an increase in pyruvate occur, and this after the 12th day of storage. The increase, however, as indicated in Fig. 4, was relatively small (less than two micromoles per milliliter) compared with the increase in the concentration of lactate (35 μM. per ml.).

A 'carbon balance' between the amount of glucose utilized and the glycolytic products formed, based on the experimental data represented in Figs. 1–4, inclusive, is given in Table I. All the data are expressed as micromoles of 3-carbon fragments per milliliter. The equivalent of the amount of glucose utilized is represented as μM. of glucose multiplied by 2. The final column (col. 8) in the table represents the difference between the total of the 3-carbon residues utilized as substrates (col. 4) and that of the reaction products (col. 7).

In the blood specimens preserved without adenosine the total of the pyruvate and lactate formed amounted to 110% of the combined amounts of glucose and other intermediates utilized. The total of the pyruvate and lactate formed in the samples containing added adenosine, on the other hand, amounted to 150% of the combined amounts of glucose and other intermediates utilized. The 40% excess represents the portion of the lactate formed from adenosine.

To study further the utilization of adenosine for the formation of lactate, the red cells from a relatively fresh blood specimen (after storage for two days at 4° C. in ACD) and also from an aged specimen (after storage for 99 days at 4° C. in ACD) were washed free from plasma with isotonic saline and finally suspended in the latter. Each specimen was then divided into aliquots, one of which served as a 'control'; the others contained glucose or adenosine, or both, as indicated in Table II. The data in the table indicate the change in the concentration of various intermediates and products of glycolysis during the two and one-half hour period at 37° C.

It is noteworthy that no significant utilization of glucose occurred in the preserved blood samples containing added sugar. Glucose was not estimated in the samples containing adenosine because of the interference of the latter with the analysis. It is safe to say, however, that no utilization of glucose had taken place in these specimens during the period.

TABLE I

CARBON BALANCE OF CHANGES IN BLOOD DURING STORAGE

Storage medium	Metabolites utilized				Metabolites produced			Balance, μM.
	(1) Glucose, μM. × 2	(2) 2,3-DPG, μM.	(3) Hexose PO_4, μM.	(4) Total, μM.	(5) Lactate, μM.	(6) Pyruvate, μM.	(7) Total, μM.	(8) = (7) − (4)
ACD	−27.2	−1.7	−0.6	−29.5	+32.0	+0.3	32.3	+ 2.8
ACD + adenosine	−33.2	+0.5	0	−32.7	+48.9	+0.3	49.2	+16.5
CD	−32.7	−1.4	−0.5	−34.6	+36.9	+1.3	38.2	+ 3.6
CD + adenosine	−37.4	+0.3	0	−37.1	+52.2	+0.3	52.5	+15.4

All values are expressed as μM. per ml. of whole blood.

TABLE II

CHANGE IN THE CONCENTRATION OF VARIOUS GLYCOLYTIC INTERMEDIATES ON INCUBATION OF WASHED ERYTHROCYTES, WITHOUT ADENOSINE (A) AND WITH ADENOSINE (B)

Each flask contained 14 ml. washed cells (made up to twice the volume of the packed cells with isotonic KCl); 0.02 *M* phosphate buffer, pH 7.3; 0.005 *M* $MgCl_2$; and 100 mgm. adenosine. Incubation: two and one-half hours at 37° C. All values are expressed as μM. per ml. of reaction mixture.

Metabolite	2-day-old blood				99-day-old blood			
	Control		With glucose		Control		With glucose	
	A	B	A	B	A	B	A	B
Glucose	+0.1	—	−0.2	—	+0.1	—	+0.1	—
Pyruvate	+0.2	0	+0.1	0	+0.1	0	+0.1	0
Lactate	−0.1	+2.6	+0.3	+2.3	−0.2	+1.2	+0.1	+0.9
P_i	+1.1	−1.5	+1.0	−1.3	+0.1	−2.2	0	−2.1
ATP	0	+0.5	0	+0.34	0	+0.2	0	+0.24
Hexose phosphates	−0.1	+0.6	−0.2	+0.6	+0.1	+0.5	−0.1	+0.5
2,3-DPG	−0.5	−0.1	−0.4	0	−0.1	+0.6	0	+0.7

No significant amount of pyruvate accumulated in any of the specimens. Relatively large amounts of lactate, on the other hand, were formed in the adenosine–containing specimens from both the fresh and the aged blood samples, whether glucose was present or not.

Considerable hydrolysis of the organic phosphate esters, chiefly of 2,3-DPG, occurred in the specimens from the fresh blood sample. These esters in the 99-day-old sample had long before been reduced to a negligible quantity. At the beginning of the incubation experiment the concentration of DPG (μ M. per ml. of reaction mixture) in the 'fresh' specimen was 0.88 μM. while that of the aged sample was 0.14 μM. At the end of the two and one-half hour period the concentration of DPG in the specimens containing adenosine remained practically unchanged (0.83 μM.) in the 'fresh'-cell specimen but had increased to 0.77 μM. in the aged-cell sample. It would appear that DPG in the human erythrocyte is in equilibrium within the glycolytic system and was present in a concentration of about 0.8–0.9 μM. under our conditions. This is in accord with the observations of Rapoport and Luebering (16, 17) that 2,3-DPG inhibits the mutase which catalyzes the interconversion of 1,3- and 2,3-DPG, and further, that 3-phosphoglycerate inhibits the phosphatase that hydrolyzes 2,3-DPG.

In accordance with the observations of Gabrio and Huennekens (10) we have found that adenosine induces a synthesis of hexosephosphates and ATP both in the fresh and the aged-cell specimens. The increase in the labile phosphate esters represents a synthesis of ATP, but may include also the formation of some labile phosphate on carbon-1 of the sugar esters. In view of the magnitude of the increase the P_L fraction could not have consisted entirely of hexose diphosphate. It must therefore represent a substantial synthesis of ATP.

The resynthesis of phosphate esters in the erythrocyte during storage of blood with added adenosine is accompanied by a pronounced reduction in the inorganic phosphate content of the cells (see Fig. 1). To ascertain whether an extreme lowering of the concentration of P_i may limit the effectiveness of adenosine in promoting resynthesis of phosphate esters, specimens were stored at 4° C., some with added inorganic phosphate and adenosine, and others with adenosine alone. As indicated in Fig. 5 a greater uptake of P_i and a more active synthesis of 2,3-DPG occurred in the former than in the latter specimens. A slight increase occurred also in the concentration of hexose phosphate but the presence of the added P_i along with the adenosine caused no change in content of ATP, in the rate of utilization of glucose, nor in the formation of lactate and pyruvate. These processes may already have been operating at a maximum rate.

As indicated in Fig. 6 there was an early rise in the pH of the specimens containing adenosine. This increase did not occur immediately upon addition of adenosine to the samples, but took place during the first few days of storage. Thereafter, the pH fell in the usual manner, owing to the formation of lactic acid. The rate of formation of the latter and hence of fall in pH was somewhat

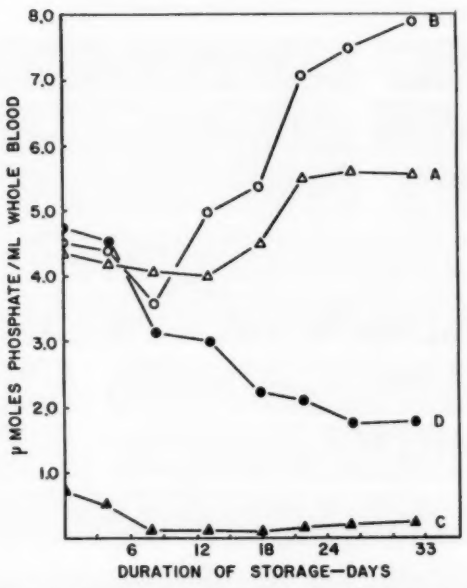

FIG. 5. Influence of added inorganic phosphate on the synthesis of 2,3-diphosphoglycerate induced by adenosine in erythrocytes preserved in ACD at 4° C.
Curve A. — DPG, without added phosphate.
Curve B. — DPG, with added phosphate.
Curve C. — P_i, without added phosphate.
Curve D. — P_i, with added phosphate.

greater in the samples containing adenosine than in the 'control' samples, although the initial pH was higher in the presence of adenosine.

The increase was presumed to arise from the progressive deamination of the adenosine, since Conway and Cook (2), several years ago, demonstrated the presence of a powerful deaminase in red blood cells. To confirm this explanation adenosine was added (final concentration 0.4%) to a specimen of blood which had been stored in ordinary ACD for two weeks at 4° C. One aliquot was maintained at 4° C. for 45 hr. while another was kept at 37° C. for nine hours. The samples had been incubated at the respective temperatures for 30 min. prior to the addition of adenosine. Periodically samples were removed for pH estimation and analysis. A portion was deproteinized with perchloric acid and the optical density of the filtrate was determined at 265 mμ in the Beckman DU spectrophotometer. The optical density at this wave length decreases as adenosine is converted to inosine (12).

Typical results are represented in Fig. 7. It may be observed that a rapid rise in pH occurred in the specimen at 37° C. reaching a maximum in about two hours. Thereafter, the pH gradually fell as the formation of lactic acid proceeded. Concurrently, the absorption at 265 mμ decreased until it reached a steady level within two to three hours.

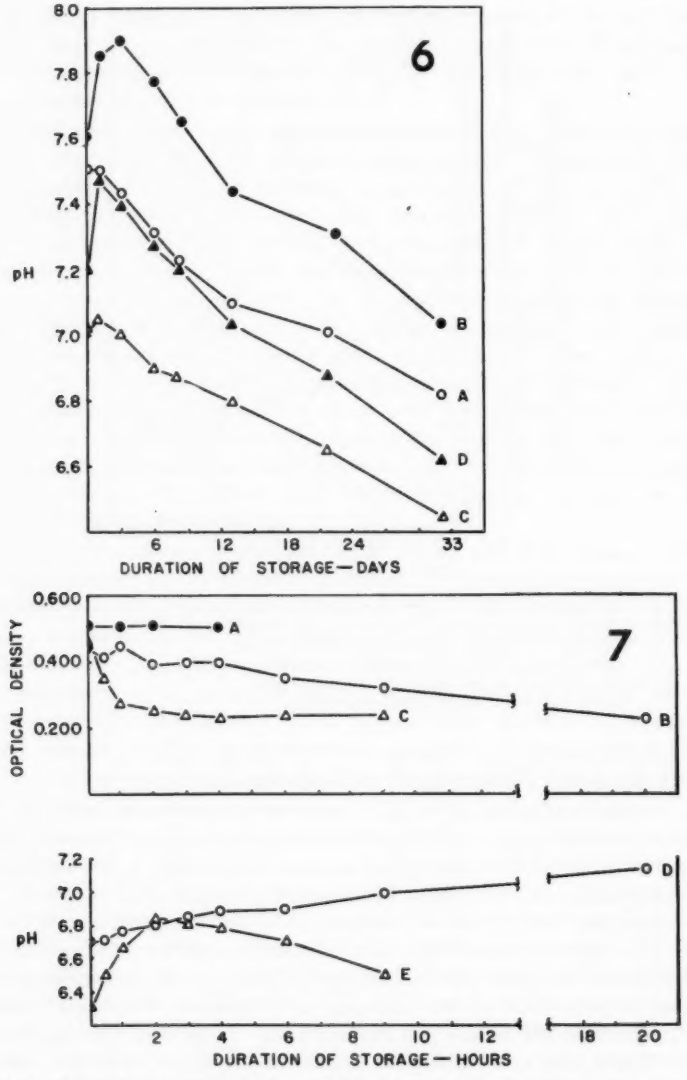

FIG. 6. The influence of adenosine on the change in pH of blood specimens stored at 4° C.

Curve A. — In CD without adenosine.
Curve B. — In CD with adenosine.
Curve C. — In ACD without adenosine.
Curve D. — In ACD with adenosine.

FIG. 7. Concurrent changes in the pH and the optical density (O.D.) at 265 mμ of blood specimens preserved in ACD in the presence or absence of adenosine at 4° C. (Curves B and D) and at 37° C. (Curves C and E). Control (Curve A): adenosine in Ringer solution at 37° C.

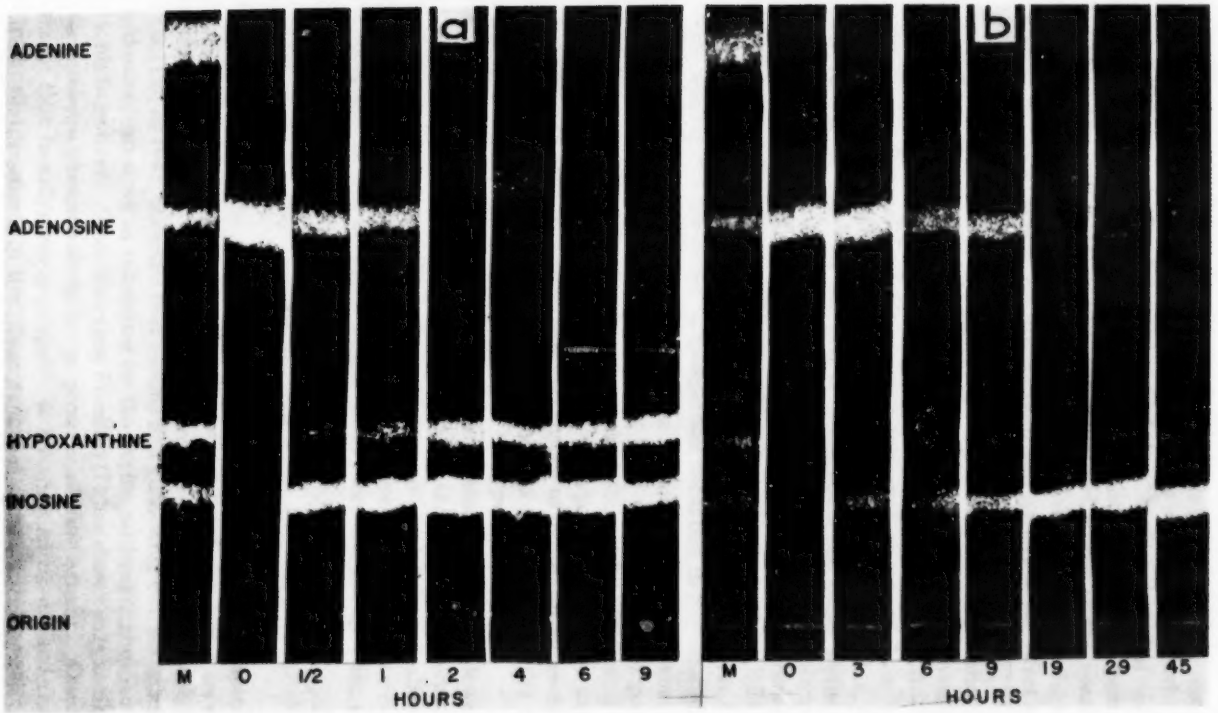

FIG. 8. Electrophoretic patterns indicating the conversion of adenosine to inosine and the splitting of the latter to hypoxanthine in blood during preservation in ACD. (M designates "marker".)

a — pattern from sample stored at 37° C. *b* — pattern from sample stored at 4° C.

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In the specimens maintained at 4° C., on the other hand, the pH continued to rise for about 19 hr. from the beginning of storage and thereafter remained constant. Concurrently, the absorption at 265 mμ steadily decreased. The absorption of the 'control' specimens suspended in 'ACD' (mixture of one part ACD and four parts of isotonic KCl) with added adenosine remained unchanged. The difference between the initial pH values of the specimens kept at 3° C. and those at 37° C. may be attributed mainly to the influence of temperature on the hydrogen ion dissociation, and partly, to the effect of the lactic acid formed during the 30 min. preincubation treatment at 37° C.

To confirm that the rapid increase in the pH arises from enzymatic deamination of the adenosine the fate of nucleoside, added to preserved blood specimens, was studied with the aid or paper ionophoresis. A 0.05 ml. quantity of the deproteinized filtrate was subjected to paper ionophoresis using a Spinco electrophoresis cell with 0.05 *M* citrate buffer, pH 2.5, and a constant voltage of 400. At the end of three hours the paper strips were dried, placed on a light-sensitive paper, and exposed to filtered ultraviolet light (240 mμ). The filter-paper strips are reproduced in the photographs shown below. Fig. 8 *a* represents the electrophoretic pattern obtained with the sample stored at 37°, and 8 *b* that at 4° C. It may be observed that adenosine was rapidly converted to inosine and the latter to hypoxanthine.

After one-half hour at 37° C. (Fig. 8 *a*) the intensity of the adenosine band was greatly diminished while a new strong band appeared at the position corresponding to that of inosine in the 'marker' strip. In addition, a faint band appeared at the position corresponding to hypoxanthine in the marker. The conversion of adenosine to inosine and thence to hypoxanthine proceeded until, at the end of two hours, the adenosine was depleted. By the ninth hour most of the inosine had been converted to hypoxanthine.

The behavior of the adenosine in the specimen stored at 4° C. followed the same pattern as that at 37° C. except that the changes occurred more slowly at the lower temperature. The adenosine was not depleted until about the 45th hour. In neither of the experiments, however, was there any evidence of the formation of free adenine. Apparently, therefore, the splitting of the nucleoside is preceded by deamination.

Discussion

It is evident from the amount of lactic acid formed in blood specimens containing added adenosine that the ribose moiety of the latter serves as precursor of lactic acid. There is ample theoretical ground for this conclusion. Kalckar (11), in 1945, observed that inosine and guanosine, but not adenosine or xanthosine, can serve as substrates for the purine nucleoside phosphorylase obtainable from liver. Since that time Friedkin (7) has shown that xanthosine also can undergo phosphorolysis in the presence of liver extract. Dische (4) has shown that hemolyzates are capable of splitting adenosine and that the subsequent disappearance of the ribose moiety is accompanied by the formation of triosephosphate, hexosediphosphate, and hexosemonophosphate. He

demonstrated also that added ribose-5-phosphate gives rise to the same products. More recently, Dische and Pollaczek (5) have demonstrated the presence of sedoheptulose in blood specimens after incubation with adenosine. Gabrio and Huennekens (10) have prepared a partially purified purine nucleoside phosphorylase from blood. According to our findings adenosine undergoes deamination; in no case was free adenine detected. The inosine is then split, presumably by phosphorolysis, yielding hypoxanthine and ribose-1-phosphate. It is of incidental interest that no hemolysis occurs despite the considerable amount of ammonia (0.015 M.) liberated within the cell during the deamination of adenosine.

The present knowledge of the relation of the metabolism of adenosine to the glycolytic system is summarized in Fig. 9.

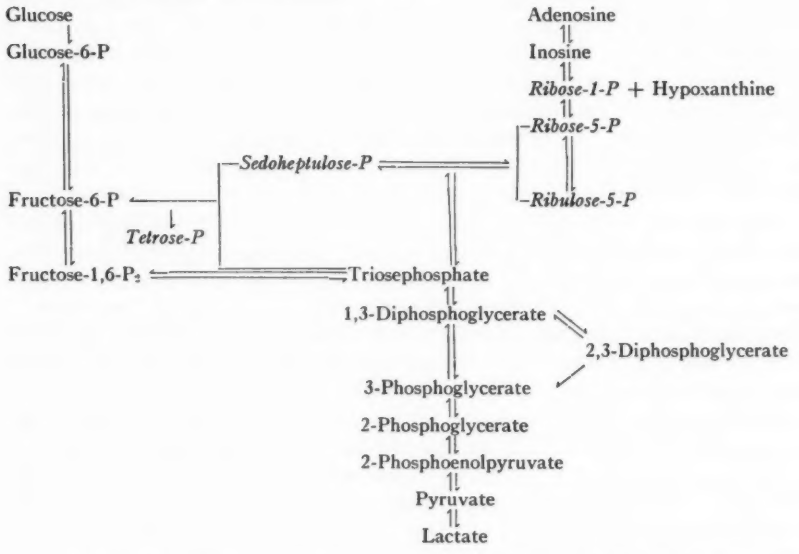

FIG. 9. Possible pathways connecting the metabolism of adenosine (ribose) and glucose in the mammalian erythrocyte.

Evidence obtained in our laboratory indicates that the failure of phosphorylation of glucose may be the primary step in the failure of the glycolytic system in the erythrocyte during storage. The above scheme indicates that, through inosine, adenosine affords a metabolic bypass to the first step of the glycolytic system. It provides the necessary intermediates for the system when the capacity of the cell to obtain these from glucose is impaired or lost. In this way the viability of the cells is maintained. Transketolase and transaldolase may play an important role in this alternative mechanism although neither of these enzymes has yet been shown, by direct methods, to be present in the red cell.

Our study indicates that the effect of added adenosine in causing a rapid decrease in the concentration of inorganic phosphate in preserved blood specimens may be accounted for by the induced synthesis of 2,3-diphosphoglycerate (DPG), and to a lesser extent, hexosephosphates. The 2,3-DPG serves as a store of readily available esterified phosphate. Even though one of the phosphate groups may be lost through hydrolysis by phosphatase during storage of blood, the phosphate on the carbon-3 of the DPG readily is utilized for the formation of ATP through the catalytic action of enolase and pyruvic phosphoferase. This agrees with the observation of Prankerd and Altman (15) who have suggested also that the shunt mechanism may be involved in the utilization of adenosine. The action of adenosine in replenishing 2,3-DPG is potentiated by the presence of an excess of inorganic phosphate in the preservative medium.

There is no evidence that glucose-6-phosphate can be converted to pentose by way of the hexosemonophosphate shunt in the erythrocyte. Hence the finding that the subsequent portion of the shunt is active is of especial interest. However, Seegmiller and Horecker (18) have shown that bone-marrow extract can convert 6-phosphogluconic acid to glucose-6-phosphate through the intermediation of pentose. The sequence of reactions would follow that shown in Fig. 9. Thus it would appear that the substrates, printed in italics in Fig. 9, represent a remnant of the hexosephosphate shunt which probably is active in a precursor of the erythrocyte.

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Addendum

Since the foregoing paper was submitted for publication a continuation of the study has indicated that inosine is as efficient as adenosine in promoting the resynthesis of phosphate esters in preserved blood specimens. Further-

more, a study* of the comparative toxicity of the two nucleosides in the dog and the rat has shown that, whereas 50 mgm. adenosine, administered by intravenous injection, caused a pronounced fall in the blood pressure in the dog, inosine in times the dose was without effect. Similarly, in the rat an injection of 10 mgm. of adenosine proved fatal whereas 200 mgm. of inosine caused no observable effect. Injection of ammonia caused a small drop in the blood pressure, but the depressor action of adenosine cannot be explained solely by the amount of ammonia liberated by deamination of the nucleoside in the blood stream (i.e. by the red blood cells).

Experiments in which blood specimens containing added adenosine or inosine were maintained at 37° C. for one and one-half hours showed that the two nucleosides were utilized at the same rate, as indicated by the rate of phosphorolysis of the compounds, the induced esterification of inorganic phosphate, and the conversion of the ribose moiety to lactate by the erythrocytes.

Blood preservation studies with inosine at lower temperatures and over longer periods of storage are in progress. If one or other of the nucleosides should be considered for the preservation of blood for clinical use, inosine is preferable to adenosine.

*We wish to acknowledge the assistance of Dr. J-M. Loiselle of our group in performing toxicity tests.

THE ENZYMATIC HYDROLYSIS PRODUCTS OF SARIN[1]

By F. C. G. Hoskin

Abstract

The hydrolysis of the powerful cholinesterase inhibitor, sarin, by a rat serum enzyme leads almost exclusively to isopropyl methylphosphonic acid, neither methylphosphonic acid nor hydrogen methylphosphonofluoridate being formed. When isopropyl methylphosphonic acid is administered to the intact rat, it is excreted unchanged in the urine. It is inferred, therefore, that the metabolism of sarin by the intact rat would lead almost exclusively to isopropyl methylphosphonic acid.

Introduction

Although the role of phosphate in lipid and other intermediary metabolism has been and continues to be a major field of investigation, little is known about the metabolism of the phosphate and phosphonate esters which comprise the so-called "irreversible" cholinesterase inhibitors. There are two reasons for this—first, the pharmacology and enzyme inhibiting properties of these compounds are of prime interest, and second, because of their extremely high toxicity it is difficult to study their metabolism and excretion in the intact animal.

The metabolism of some organophosphorus cholinesterase inhibitors by plants and animals is directly related to their toxicity as systemic insecticides (13). Although the reactions are not clearly known, they appear to be more complex than one of hydrolysis. Many phosphate and phosphonate esters, while slowly decomposed in the presence of water, are hydrolyzed even more rapidly by animal enzymes. The formation of acids, as from diisopropyl phosphorofluoridate (DFP) (12), or of colored compounds, as from diethyl *p*-nitrophenyl phosphate (paraoxon, E600) (1), has often facilitated the study of these reactions.

The work of Augustinsson and Heimbürger (3, 4) and Hoskin and Trick (8) indicates that the *in vitro* enzymatic hydrolysis of ethyl N,N-dimethylphosphoramidocyanidate (tabun) leads almost exclusively to cyanide and ethyl N,N-dimethylphosphoramidic acid. Over long periods of time and at more acid pH, the aqueous hydrolysis of tabun proceeds even further to yield dimethylamine and the monoethyl ester of phosphoric acid (11).

In the present work, the metabolism of the powerful cholinesterase inhibitor (6) and highly toxic compound (10), isopropyl methylphosphonofluoridate (sarin), has been investigated. The principal and almost exclusive rat serum enzymatic hydrolysis product has been determined, as well as, by indirect evidence, the principal and almost exclusive urinary excretion product of sarin from the intact rat.

[1] *Manuscript received September 23, 1955.*
Contribution from the Physiology Section, Defence Research Board, Suffield Experimental Station, Ralston, Alberta.

Materials and Methods

Sarin, both non-radioactive and labelled with P^{32}, was supplied through the courtesy of Mr. B. J. Perry* as were also methylphosphonic acid, hydrogen methylphosphonofluoridate†, and isopropyl methylphosphonic acid. This latter compound labelled with P^{32} was made by the hydrolysis of sarin-P^{32} and purified by ether extraction and distillation (80° C. at 0.008 mm. Hg).

The source of enzyme was pooled serum from seven or more adult male Wistar rats. This strain was also used for the *in vivo* experiment.

The silver salt of isopropyl methylphosphonic acid was prepared by warming a dilute solution of the acid with freshly prepared silver oxide. The solubility of the product in hot water permitted its separation from the reaction mixture. The silver salts of methylphosphonic acid and hydrogen methylphosphonofluoridate were made by the addition of silver nitrate to dilute solutions of the acids nearly neutralized with ammonia (7). All silver salts were dried *in vacuo*.

Radioactive measurements were made on thin samples evaporated onto aluminum discs, using a Geiger–Müller type end-window tube and conventional recording apparatus. The values recorded in Tables I and II have been obtained by an application of the isotope dilution principle (9). In addition to the usual corrections involved in the measurement of radioactivity, these values have been corrected for differences in molecular weight and volumes of reaction mixture used.

Experimental and Results

Sarin-P^{32} (70 mgm.) was incubated for nine hours at 38° C. in 100 ml. of 0.02 *M* sodium bicarbonate containing 2 ml. of rat serum, the cholinesterase of which had previously been inhibited *in vivo* by the administration of about 0.02 mgm. of non-radioactive sarin per rat. At the end of the incubation period 0.2 ml. of the reaction mixture was injected intraperitoneally into each of five 25 gm. female mice. None of the animals died, indicating that more than 90% of the sarin had been destroyed.

One-half of the reaction mixture was continuously extracted with ether for 17 hr., during the first hour of which time 10 gm. of non-radioactive isopropyl methylphosphonic acid was metered into the aqueous phase in the liquid–liquid extraction apparatus. At the end of the extraction period the ethereal solution was evaporated under reduced pressure and the remaining oily liquid was distilled. The middle of three fractions, all collected at 80° C. and 0.008 mm. Hg, was converted to the silver salt of isopropyl methylphosphonic acid.

Of the remaining half of the reaction mixture, a portion was extracted twice with ether, 4.0 gm. of solid methylphosphonic acid was added, the solution

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† *This acid exists in equilibrium with about five per cent of its disproportionation products (14).*

was neutralized with ammonia, and precipitation was completed with silver nitrate. This precipitate, silver methylphosphonate, was washed several times with hot water before being dried.

Non-radioactive hydrogen methylphosphonofluoridate (4.0 gm.) was added to a second ether-extracted portion of the reaction mixture, the solution was neutralized with ammonia and precipitation was completed with silver nitrate. This precipitated silver salt was washed several times with hot water before being dried.

Trichloroacetic acid was added in the cold to a third portion of the reaction mixture to the extent of 10%. The precipitated proteins were washed repeatedly with cold water and were finally taken up in 0.1 *N* alkali.

The results of radioactivity measurements on the three silver salts, on the precipitated protein, and on the aqueous solution remaining from the continuous ether extraction are shown in Table I.

Two 200 gm. rats were given a total of 285 mgm. of P^{32}-labelled isopropyl methylphosphonic acid over a period of 48 hr. The acid was dissolved in a buffered aqueous medium and injected subcutaneously. About 15 ml. of urine was collected over a period of 72 hr. and was made up to 50 ml. with distilled water.

One-half of this solution was continuously extracted with ether for 17 hr., during the first hour of which time 3.24 gm. of non-radioactive isopropyl methylphosphonic acid was metered into the aqueous phase in the liquid–liquid extraction apparatus. The acid was recovered and converted to its silver salt by the operation previously described.

Of the remaining half of the urine solution, 20 ml. was evaporated to dryness, 0.69 gm. of non-radioactive methylphosphonic acid was added to the flask, and the tarry contents were shaken for one hour with absolute

TABLE I

FATE OF RADIOACTIVE SARIN FOLLOWING HYDROLYSIS BY RAT SERUM

Fraction	Total radioactivity, counts/min.	% added radioactivity
Added sarin	13.01×10^8 (± 1.9%)	100
Ag salt of isopropyl methylphosphonic acid	12.1×10^8 (± 2.8%)	93
Ag salt of methylphosphonic acid	0.19×10^8 (± 2.5%)	1.5
Ag salt of hydrogen methylphosphonofluoridate	No activity above background	0
Precipitated protein	4.4×10^8 (± 1.8%)	0.3
Residue	0.98×10^8 (± 6.4%)	7.5
Total accounted for		102.3

TABLE II

FATE OF RADIOACTIVE ISOPROPYL METHYLPHOSPHONIC ACID IN THE INTACT RAT

Fraction	Total radioactivity, counts/min.	% excreted radioactivity
Administered isopropyl methylphosphonic acid	18.7×10^7 (± 2.4%)	*
Excreted urine	10.8×10^7 (± 4.0%)	100*
Ag salt of isopropyl methylphosphonic acid	9.2×10^7 (± 1.4%)	85.1
Ag salt of methylphosphonic acid	3.0×10^5 (± 27%)†	0.3
Residue	1.2×10^7 (± 2.9%)	11.1
Total accounted for		96.5

** The total radioactivity excreted in the urine has been arbitrarily set equal to 100%. It is actually 57.7% of the administered radioactivity.*

† The radioactivity of these samples was extremely low (15 ± 4 counts/min.), hence the high standard deviation (27%).

ethanol. An equal volume of ether was then added, the mixture was filtered, the filtrate was evaporated, and the residue was taken up in water. This acid solution was neutralized with ammonia and precipitation was completed with silver nitrate. The precipitate, silver methylphosphonate, was washed several times with hot water before being dried.

The results of radioactivity measurements on the excreted urine, on the two silver salts, and on the aqueous solution remaining from the continuous ether extraction are shown in Table II.

Discussion

The first experiment (Table I) shows that the enzymatic hydrolysis of sarin by rat serum leads almost exclusively to isopropyl methylphosphonic acid with little or no methylphosphonic acid being formed.

In Table I and elsewhere in this paper reference has been made to the silver salt of hydrogen methylphosphonofluoridate. What was obtained was probably the silver salt of methylphosphonic acid. Infrared absorption spectra indicate that hydrogen methylphosphonofluoridate disproportionates in the following manner (14):

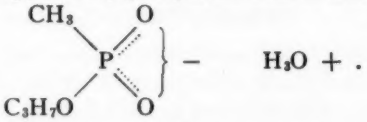
$$2\ CH_3POF(OH) \rightleftharpoons CH_3POF_2 + CH_3PO(OH)_2 .$$

Therefore the entries in Table I entitled "Ag salt of methylphosphonic acid" and "Ag salt of hydrogen methylphosphonofluoridate" both probably refer to the same compound. However, no confusion arises since there was little or no radioactivity in either case.

Because of the extreme toxicity of sarin, it is practically impossible to determine the course of its metabolism in the intact animal. The results shown in Table II, however, indicate that, following the administration of the

principal *in vitro* enzymatic hydrolysis product (isopropyl methylphosphonic acid) to the intact rat, the administered compound itself is excreted unchanged. Although some 40% of the administered compound had not been excreted in the urine at the end of 72 hr., it is unlikely that any appreciable amount of the 40% would be further hydrolyzed but not excreted. The results indicate that the half-acid half-ester, isopropyl methylphosphonic acid, is not hydrolyzed in the intact rat to dibasic methylphosphonic acid. This is a rather surprising observation in view of the many esters which are hydrolyzed either in the animal body or by fractions of animal tissues and fluids (2, 5).

The following structural formula is given to illustrate the electronic properties of isopropyl methylphosphonic acid in water:



The resonance stabilization of the isopropyl methylphosphonate ion is immediately evident and is offered as an explanation for the resistance of this compound to further hydrolysis.

Acknowledgment

I should like to thank Dr. M. K. McPhail, Head of the Physiology Section, for his continued advice and encouragement.

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KINETICS OF THE ENZYMATIC HYDROLYSIS OF SARIN[1]

By P. A. Adie, F. C. G. Hoskin, and G. S. Trick

Abstract

The enzymatic hydrolysis of sarin is apparently a single first-order reaction. There is no evidence of different reaction rates for the two possible optical isomers of sarin. During both the enzymatic and the non-enzymatic hydrolyses, sarin appears to be detoxified somewhat more rapidly than the manometric results would indicate. However, the detoxification parallels the manometric results sufficiently to stand in contrast to results obtained using tabun.

Introduction

In the preceding paper (2) isopropyl methylphosphonic acid was shown to be the exclusive phosphorus-containing product of the enzymatic hydrolysis of sarin (isopropyl methylphosphonofluoridate). Hydrofluoric acid must be, perforce, liberated in equimolar quantity. Augustinsson and Heimbürger (1) made passing reference to the enzymatic hydrolysis of sarin in their report of the enzymatic hydrolysis of tabun. Sarin, as well as tabun, should be capable of existing in two optically active forms. This has been pointed out and supported experimentally by Michel (5). Evidence has also been presented for the existence of optical isomers of tabun (3).

The present investigation was undertaken in order to examine the kinetics of the enzymatic hydrolysis of sarin. The results, while not in conflict with those of Michel (5), are somewhat unexpected in view of our results with tabun (3).

Materials and Methods

Freshly distilled sarin was dissolved in distilled water immediately before use. The source of enzyme was serum from seven or more adult male Wistar rats whose cholinesterase had been inhibited *in vivo*. The serum was diluted as indicated with 0.85% saline solution.

The hydrolysis of sarin was followed manometrically in the Warburg apparatus at 38° C. ± 0.1°. The main compartment of the reaction flasks contained 0.5 ml. of diluted serum and 4.0 ml. of 0.025 *M* sodium bicarbonate equilibrated with 95% nitrogen: 5% carbon dioxide. For non-enzymatic hydrolysis, the serum was replaced by 0.5 ml. of 0.85% saline solution. The side arm contained 0.5 ml. of 0.05 *M* sarin solution (hence 0.005 *M* after tipping).

The hydrolysis, both enzymatic and non-enzymatic, was repeated at the same concentrations but in larger quantity. At time intervals the toxicity of the reaction mixture was tested intraperitoneally using female mice. By

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Contribution from the Physiology and Chemistry Sections, Defence Research Board, Suffield Experimental Station, Ralston, Alberta. Presented to the Western Regional Group, Division of Medical Research, National Research Council of Canada, in Edmonton, February 17, 1955.

determining initially the toxicity of sarin and by using the familiar logarithm-dose versus probability-response plot (4) it was possible to estimate the percentage of sarin destroyed at various times.

Results and Discussion

Fig. 1 shows the evolution of carbon dioxide during both the enzymatic and the non-enzymatic hydrolysis of sarin in the presence of a bicarbonate buffer. The data have been plotted on a logarithmic scale, i.e., as first-order reactions. The four lines depict, from top to bottom, the hydrolysis of sarin in the absence of rat serum (control) and in the presence of rat serum diluted (*a*) 1 to 5, (*b*) 2 to 5, and (*c*) 4 to 5. The theoretical gas yield for this reaction is 1278 mm.[3] The reactions illustrated by curves (*a*) and (*b*) were continued until they were 80% and 75% complete, respectively. The non-enzymatic hydrolysis was disctontinued for reasons of time economy and the reaction shown by curve (*c*) became erratic in all determinations after about 100 min.

In Fig. 2 the first-order reaction constants calculated from Fig. 1 are plotted against the enzyme (serum) concentration. The direct linear relationship shown over this range is a further indication that the hydrolysis of sarin, both enzymatic and non-enzymatic, is a single first-order reaction under the present conditions.

Fig. 3 shows the rate of disappearance of toxicity during the hydrolysis of sarin in the absence of rat serum and in the presence of rat serum diluted 1 to 5, as compared to the rate of carbon dioxide evolution under the same circumstances. During about the first 100 min., the amount of sarin destroyed appears to be the same whether measured manometrically or toximetrically. From about this point onward, there is an ever increasing discrepancy between

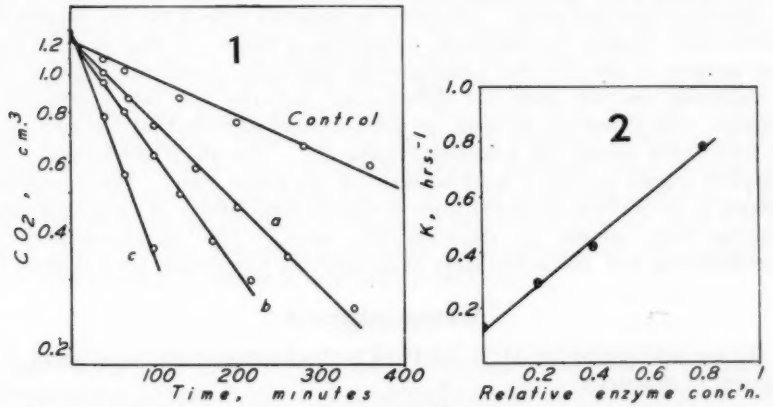

FIG. 1. Carbon dioxide evolution during the hydrolysis of sarin in a bicarbonate buffer. The data are plotted on a logarithmic scale (ordinate), i.e., as first-order reactions. (*a*) Rat serum diluted 1 to 5, (*b*) rat serum diluted 2 to 5, (*c*) rat serum diluted 4 to 5 and (control) saline added in place of rat serum.

FIG. 2. The first-order reaction constants (ordinate) plotted against relative enzyme concentrations. Undiluted rat serum = 1.

the apparent percentages of hydrolysis as measured by the two methods. This discrepancy has not been found amenable to any experimentally demonstrable explanation. Nevertheless, these results stand in contrast to the work with tabun (3) wherein that compound had been almost completely detoxified when the manometric data indicated that it had been only half hydrolyzed.

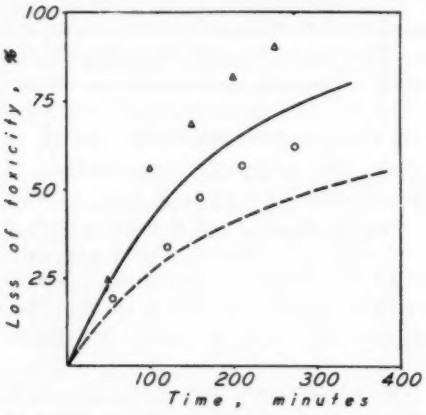

FIG. 3. The rate of destruction of sarin as estimated manometrically and by toxicity tests on mice. The manometric data have been taken from Fig. 1: continuous line, enzymatic (rat serum diluted 1 to 5); dashed line, non-enzymatic. The toximetric data are shown by discrete points; △ enzymatic (rat serum diluted 1 to 5); ○ non-enzymatic.

These results indicate that the enzymatic hydrolysis of sarin is kinetically a reaction of the first order. The same is probably true of the non-enzymatic hydrolysis. Unlike the enzymatic hydrolysis of tabun (3), that of sarin does not appear to involve two simultaneous first-order reactions. Either the hydrolyzing enzyme does not differentiate between the two theoretically possible optical isomers of sarin, or differentiates so little that the two rates of hydrolysis cannot be resolved graphically. The slight deviations from linearity shown in Fig. 1 tend to support the latter view. There appears, therefore, to be little or no evidence of the stereospecificity of the hydrolyzing enzyme with respect to its substrate, sarin. The stereospecificity of cholinesterase with respect to sarin as an inhibitor has already been shown (5).

Acknowledgment

We are indebted to Dr. M. K. McPhail for his continued encouragement and interest in this work.

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CLEAVAGE OF CELLOTRIOSE BY MYROTHECIUM CELLULASE[1]

By D. R. Whitaker and E. Merler[2]

Abstract

The method of cleavage of cellotriose by *Myrothecium* cellulase was determined from the distribution of C^{14} in the enzymic hydrolysis products of cellotriose labelled in the anhydroglucose unit at the reducing end of the chain. The substrate was prepared by the incubation of the enzyme in a concentrated solution of cellopentaose (0·14 *M*) and C^{14}-labelled glucose (5 *M*) and the fractionation of the products, first by adsorption and elution on a charcoal column and then by preparative paper chromatography. The glucosidic linkage adjacent to the non-reducing end of the chain is preferentially hydrolyzed by the enzyme. Its rate of hydrolysis was estimated to be about five times that for the linkage adjacent to the reducing end of the chain.

Introduction

One of the trends shown in the hydrolysis of β-1:4′-oligoglucosides by *Myrothecium* cellulase is that as the number of anhydroglucose units in the oligoglucoside chain is increased from four to six, the method of cleaving the chain becomes more random (10). The composition of hydrolyzates of cellotetraose, the sugar with a chain of four anhydroglucose units, indicates that cleavage of the central glucosidic linkage of the chain is favored over cleavage at the two terminal linkages; the relative frequency of cleavage at the central linkage was estimated to be about 0·6, i.e., nearly twice the relative frequency (0·33) predicted on the assumption of random cleavage. Cleavage of the non-terminal linkages of cellopentaose is also favored, although at least one of the terminal linkages is cleaved at a higher rate than either of the terminal linkages of cellotetraose. Hydrolyzates of cellohexaose show little evidence of preferential cleavage at one linkage; several linkages of the chain appear to be more or less equally susceptible to hydrolysis.

It might be expected from this trend that the substrate cleaved with the greatest selectivity would be cellotriose, the sugar with a chain of three anhydroglucose units. However, the method of cleavage cannot be determined by analysis of hydrolyzates of the naturally occurring sugar, as cleavage at either of its two linkages gives rise to the same hydrolysis products. This difficulty does not arise if the substrate is selectively labelled with C^{14} at either of its terminal anhydroglucose units. The preparation and method of cleavage of such a substrate is described in this report.

Materials

The enzyme was obtained from culture filtrates of the mold *Myrothecium verrucaria* and purified by methods previously described (9). Cellotriose and

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cellopentaose were obtained from an acetolyzate of cellulose by fractionation, after deacetylation, on charcoal columns (10). D-Glucose, uniformly labelled with C^{14}, was obtained from Atomic Energy of Canada Ltd.

Methods and Results

1. *General Procedures*

The chromatographic fractionations were carried out on composite papers, each consisting of a large sheet (57 × 22 cm.) of Schleicher and Schuell No. 598 paper with a wick of Whatman No. 1 paper (an 11 × 22 cm. strip sewed to the upper edge of the Schleicher and Schuell paper) to reduce the rate of solvent flow (10). The sample was distributed along the central 10 cm. of the base line with mixtures of the reference sugars on either side. A multiple development procedure was used throughout, i.e., the chromatograms were developed with one solvent, dried in a current of air, and then redeveloped with the same or another solvent (3). The developing solvents were either the organic phase of a mixture of isoamyl alcohol, pyridine, and water (1:1:1 v/v) or a mixture of butanol, ethanol, and water (5:4:1 v/v). These solvents will be designated as I–P–W and B–E–W respectively. After development and drying, the papers were scanned along their lengths with a Geiger–Müller monitor mounted above a shield with a 1·5 cm. slit. The side strips containing the reference compounds and a narrow strip from the middle of the paper were cut out and sprayed with alkaline silver nitrate (6) to indicate the sugars and their derivatives. The rest of the paper was sectioned accordingly. The sugars in each section were eluted with water by a procedure previously described (10) and the eluates evaporated to dryness in a modified Craig evaporator (2). If required for C^{14} assay, the residue was dissolved in water, transferred to a platinum boat, evaporated nearly to dryness on a steam plate, and dried in an oven at 110°C.

Carbon-14 in the eluted samples was assayed after dry combustion and precipitation as barium carbonate (1). The precipitate was collected on tared millipore filters which had been cut to fit the filter unit and the mounting unit of the counter. The precipitate was dried at 110°C. with a brass ring placed over the filter to prevent curling during drying. The counting rate was measured with a thin window Geiger–Müller counter and corrected for background and to zero self absorption.

2. *Enzymic Synthesis of C^{14}-labelled Cellotriose*

The synthesis of labelled cellotriose was first attempted by the incubation of the enzyme with cellobiose (2% w/v) and labelled glucose (1% w/v). Trace amounts of cellotriose were formed under these conditions and, after the addition of unlabelled cellotriose as carrier and the fractionation of the products on a charcoal column by the method of Whistler and Durso (7), a product was recovered which gave a fairly high count on assay for C^{14}. However on subsequent chromatographic fractionation, the count fell to only a few counts above background. The product responsible for the initial activity

was probably an oligoglucoside of the amylose series as the preparation of labelled glucose used in the experiment was subsequently found to contain small amounts of maltose and several higher oligoglucosides.

The method finally adopted was to incubate the enzyme with cellopentaose in the presence of a high concentration of purified, labelled glucose. A similar procedure has been used by Rees (5) to prepare labelled amylotriose from amylopentaose and glucose.

To purify the labelled glucose a mixture of labelled glucose (3·1 mgm. containing 200 μc. of C^{14}) and reagent-grade glucose (100 mgm.) was distributed over four chromatograms and developed for 22 hr. with I–P–W and 21 hr. with B–E–W. This development advanced the glucose front from 35 to 40 cm. From the sections cut between the glucose front and a line 8 cm. behind it, 95 mgm. of glucose was recovered on elution.

Purified glucose (95 mgm.), cellopentaose (11·7 mgm.), and enzyme (50 μgm. protein) were dissolved in 1·0 ml. of buffer (0·04 *M* with respect to sodium chloride, 0·01 *M* with respect to sodium acetate, adjusted to pH 5·0 with acetic acid) and incubated for one hour at 38·9°C. Two milliliters of ethanol were then added and the solution boiled for five minutes to denature the enzyme. After vacuum evaporation to remove ethanol, the solution was made up to 25 ml. with water. Unlabelled cellotriose (51 mgm.) and cellobiose (10 mgm.) were added to the solution, the former as a carrier, the latter as a marker for the subsequent fractionation.

The solution was added in small portions, each followed by an equal volume of water, to a 7 × 2·5 cm. column of Darco-G-60 charcoal mixed with Celite 535 (7). The progress of elution from the column was followed by spot tests on the eluate with a solution of anthrone in concentrated sulphuric acid. The column was washed with 250 ml. of water to remove glucose, and then with 325 ml. of 7·5% (v/v) aqueous ethanol and 100 ml. of 10% (v/v) ethanol to remove cellobiose. Cellotriose was eluted with 400 ml. of 15% (v/v) ethanol. The eluate was evaporated to dryness to give a residue weighing 50 mgm.

The residue was distributed over two chromatograms which were developed for 22 hr. with I–P–W, then for 21 hr. with I–P–W, and finally for 19 hr. with B–E–W. The reference compounds in the side strips showed the following average migrations: glucose, 48 cm.; cellobiose, 31 cm.; cellotriose, 18 cm. The strips cut from the middle of the chromatogram showed only one component, coinciding in position with cellotriose. Scanning with the monitor showed a minor zone of C^{14} at the glucose front (maximum count: 20 c.p.m. above background) and a major zone coinciding in position with cellotriose (maximum count: 300 c.p.m. above background). A strip 6 cm. wide was cut from each sheet with its leading edge at the cellotriose front. It was eluted with water, evaporated to dryness, triturated with ethanol, and dried in vacuum.

As a check on the homogeneity of the product, two 0·5 mgm. samples were developed on paper chromatograms; one for 24 hr., then for 22 hr. with I–P–W, the other for similar periods with B–E–W. After drying, the chromatograms

were left for one week in contact with Kodak No Screen X-ray film. The chromatograms were then sprayed to indicate the sugars. The radioautographs and tracings of the sprayed chromatograms are compared in Fig. 1.

The dried product (44 mgm.) had a specific activity of 1195 c.p.m./mgm. The total activity corresponds to an incorporation of roughly 0·3% of the labelled glucose to cellotriose.

3. *Distribution of C^{14} in the Labelled Cellotriose*

The distribution of C^{14} was determined by oxidizing cellotriose to cellotrionic acid, hydrolyzing the product to glucose and gluconic acid, and assaying the products after separation by paper chromatography. The procedure distinguishes between glucose at the reducing end of the chain, which appears as gluconic acid after hydrolysis, and glucose in the two other anhydroglucose units of cellotriose.

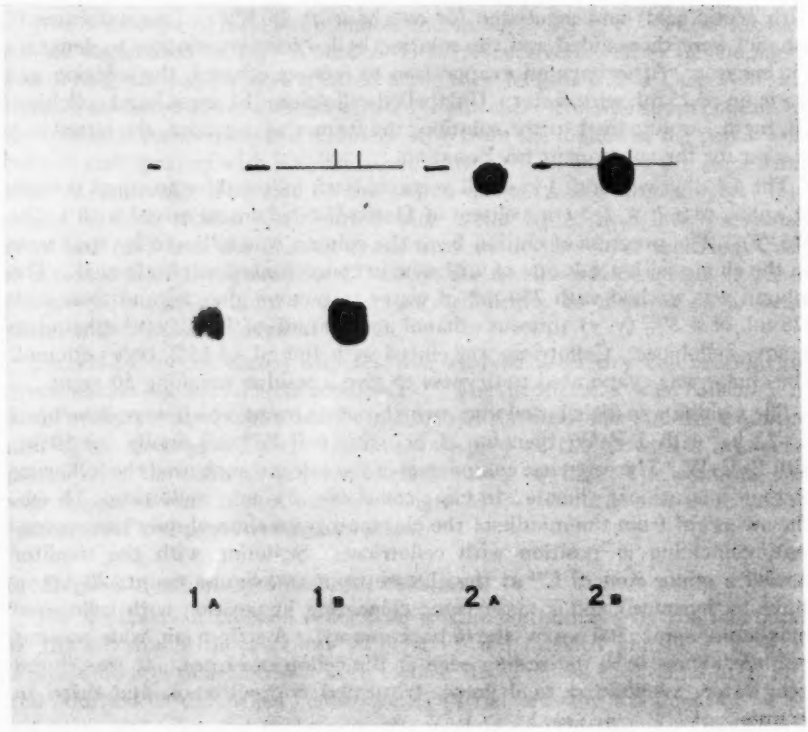

FIG. 1. Comparison of radioautographs (*A*) with tracings of sprayed chromatograms (*B*) after chromatographic development of labelled cellotriose with I–P–W (1) and B–E–W (2).

A mixture of labelled cellotriose (5 mgm.) and unlabelled cellotriose (5 mgm.) was dissolved in 0·5 ml. of water and oxidized to cellotrionic acid with a buffered solution of iodine by the method of Whistler and Tu (8). In the subsequent titration with sodium thiosulphate, the reduction of excess iodine was followed by spot tests on starch-paper. The solution was then adjusted to pH 3 and added to a column of Darco-G-60 mixed with Celite 535. The column was washed with a large volume of water to remove inorganic salts and then with increasing concentrations of aqueous ethanol. Cellotrionic acid was eluted with 20% ethanol. The eluate was evaporated to dryness (weight of residue: 11 mgm.).

The residue was developed on a paper chromatogram for 54 hr. with I–P–W and 34 hr. with B–E–W. At the end of this time, cellotriose in the side strip had migrated 16 cm., cellotrionic acid, 4·2 cm. A strip cut from the middle of the paper showed only one component, coinciding in position with cellotrionic acid. The only zone giving an appreciable count on the monitor was in the same region. A strip 3 cm. wide was cut from this region and eluted with 25 ml. of water.

An aliquot of the eluate was evaporated to dryness for direct combustion and assay. A second aliquot was acidified to a hydrochloric acid concentration of 0·2 *N* and autoclaved for 45 min. at a steam pressure of 15 p.s.i. The hydrolyzate was repeatedly concentrated by vacuum distillation, diluted with water, and reevaporated, in order to remove most of the hydrochloric acid. It was then evaporated to dryness and left overnight in a vacuum desiccator containing potassium hydroxide. The residue was developed on a chromatogram for 18 hr. with I–P–W and for 15 hr. with B–E–W. A strip cut from the middle of the paper showed only two components: one, 33 cm. from the base line, coinciding in position with glucose in the side strip; the other, 8 cm. from the base line, coinciding in position with gluconic acid in the side strip. The strips cut from these regions were eluted with water and assayed for C^{14}.

The C^{14} assays for the aliquots were as follows:

Before hydrolysis	1861 c.p.m.
After hydrolysis	
Glucose fraction	61 c.p.m.
Gluconic acid fraction	1742 c.p.m.

The results indicate that C^{14} in the labelled cellotriose was confined almost entirely to the anhydroglucose unit at the reducing end of the chain.

4. *Enzymic Hydrolysis of Labelled Cellotriose*

The procedure consisted of partial enzymic hydrolysis at a low substrate concentration, chromatographic fractionation of the hydrolyzate, and assay for C^{14} of the separated components.

A mixture of labelled cellotriose (4·8 mgm.) and unlabelled cellotriose (5·2 mgm.), dissolved in 33·3 ml. of a solution of 0·0125 *M* zinc sulphate which had been adjusted to pH 5·0 with hydrochloric acid, was incubated with enzyme (20 μgm. protein/ml. of solution) for 30 min. at 38·9°C. The increase

in reducing sugar end groups, as shown by Nelson analyses (4) of 1·0 ml. samples withdrawn at the start and at the end of the incubation period, corresponded to about 60% hydrolysis of the substrate. The solution was then brought to pH 8·0 with barium hydroxide and left for 30 min. at room temperature before it was centrifuged. The sediment of zinc hydroxide and barium sulphate was dispersed in 5 ml. of water and recentrifuged. The combined supernatant solutions were concentrated by vacuum evaporation and developed on a chromatogram for 24 hr. with I–P–W and 16 hr. with B–E–W. The chromatogram showed three components, corresponding in position to cellotriose, cellobiose, and glucose. Strips were cut accordingly and eluted with water.

The C^{14} assays gave the following results:

Total counts /min. in	
Cellotriose	1786
Cellobiose	2576
Glucose	512
	4874

The total recovery is about 93% of the theoretical recovery calculated from the weight of labelled cellotriose used for the hydrolysis.

On the basis of the C^{14} assays, the rate at which cellotriose is degraded by cleavage at the linkage adjacent to the non-reducing end of the chain is about five times the rate for cleavage at the linkage adjacent to the reducing end of the chain. In principle, the estimate should be corrected for glucose formed by hydrolysis of cellobiose during the period of enzymic hydrolysis of cellotriose. The correction would tend to increase the estimated ratio of the two rates. However the correction would be small—under the conditions chosen, the rate of enzymic hydrolysis of cellobiose is extremely low (10)—and probably within the experimental error of the assay and fractionation procedures.

Discussion

No attempt was made to determine the mechanism of formation of cellotriose from glucose and cellopentaose. Possible mechanisms include:

(1) Reversal of a hydrolytic reaction to give cellohexaose, followed by cleavage to cellotriose

$$\text{cellopentaose} + \text{glucose} \rightarrow \text{cellohexaose} \rightarrow 2\ \text{cellotriose}.$$

(2) A direct transfer reaction

$$\text{cellopentaose} + \text{glucose} \rightarrow 2\ \text{cellotriose}.$$

Neither type of reaction could be expected to give rise to labelled cellotriose exclusively and some indication of the presence of other labelled oligoglucosides was obtained during the subsequent fractionation.

The high degree of selectivity shown in the cleavage of cellotriose by *Myrothecium* cellulase is in accordance with the trend shown in its hydrolysis of higher oligoglucosides. Other aspects of the results will be discussed in a subsequent paper.

Acknowledgment

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SEASONAL RECRUDESCENCE OF THE THYMUS IN ADULT BIRDS[1]

By E. O. Höhn

Abstract

The thymus of adult mallards and house sparrows of both sexes and of female robins shows a marked enlargement following the annual breeding season. In male robins the thymus begins to enlarge earlier, almost synchronously with the testes. Histologically the enlarged thymus becomes indistinguishable from the uninvoluted thymus of immature birds, showing lobulation and development of a cortex and medulla. In the atrophic thymus found in adults for most of the year these two zones are not recognizable. The bursa Fabricii of adults does not enlarge at any time. Evidence of a similar thymus enlargement in some other species of birds is presented. The distribution of thymus tissue in passerine birds and hawks differs from the well known arrangement in the chicken; the passerine pattern is illustrated. It was not possible to bring about thymic enlargement out of season by castration or unilateral adrenalectomy in mallards.

Introduction

In an earlier investigation (3) it was shown that the thymus of adult mallards (*Anas platyrhynchos*) collected in London (England) parks undergoes a marked enlargement shortly after the end of the breeding season. The period of thymus enlargement lasted about four weeks. During this time thymus weights were about ten times those observed at other times of the year and the thymus became histologically indistinguishable from that of immature birds. It was decided to repeat observations on seasonal changes in thymus weight and histology in wild Alberta mallards and to include the bursa of Fabricius in the study. The bursa like the thymus is well developed in immature birds and undergoes involution with the onset of puberty, and it seemed desirable to determine whether the postbreeding season thymus enlargement also affected the bursa. Similar observations were also carried out on house sparrows (*Passer domesticus*) and robins (*Turdus migratorius*).

Material and Methods

During the years 1949–1954, 30 adult males, 11 juvenile males, 17 adult females, and 7 juvenile female mallards were shot in the Edmonton district (except 3 killed in southern Alberta near Medicine Hat). The series also includes some birds from a wintering flock at Calgary which were first shipped to Edmonton and were killed after having been kept in the Edmonton zoo for some weeks. Birds which had thus been kept captive for some time before they were killed are indicated in the graphs with the letter "z".

During the same period 31 adult male, 8 juvenile male, 20 adult female, and 6 juvenile female house sparrows were also collected, all in the Edmonton district. Robins were collected in the period May–October, 1954, and April,

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Contribution from the Department of Physiology and Pharmacology, University of Alberta, Edmonton, Alberta. This work was supported by a grant from the Medical Research Fund of the University of Alberta.

1955, also in the Edmonton district, 18 adult males, 8 juvenile males, 11 adult females, and 7 juvenile females being obtained. Some of the robins were fairly heavily parasitized with cestodes in the abdominal cavity; individuals so affected are indicated "W" in the graphs.

In all cases the birds were weighed and the thymus, bursa of Fabricius, gonads, and oviduct or vas deferens and seminal glomus (this was omitted in the mallards) dissected out and preserved in 10% Formol–saline. After about 16 hr. fixation, the tissues were blotted on filter to remove excess moisture and they were then weighed. Paraffin sections were prepared from thymi, bursae, and testes and stained with haematoxylin–eosin. It should perhaps be pointed out that in the three species under consideration the individual reaches full sexual maturity in the spring of the year following the year of birth.

Results

Weights of Thymus and Bursa

Mallards

In Fig. 1 the seasonal weights of the thymus and of both testes of adult male mallards are shown graphically. The period during which mature spermatozoa were observed in the testicular sections is also indicated. Fig. 1 indicates that thymus weights are higher during August and September than at other times. The mean thymus weight for the seven birds collected during August and September is 556·6 mgm., while for the rest of the year the mean thymus weight is only 108·5 mgm. This difference is statistically significant giving a t value of 5·609, $P = 0{\cdot}01$. The mean thymus weight of 11 juvenile males collected between late July and early November was 800 mgm.

The bursa Fabricii of the adult males had an average weight of 37 mgm., and there was no indication of an enlargement of this structure at any season. Average weight of the bursa of the juvenile males was 798 mgm.

The weights of thymus and oviduct of adult female mallards in the course of the year are shown in Fig. 2 as a graph. Again there is evidence of a postbreeding season increase in thymus weight, involving the period July–September. Mean thymus weight during this period was 526 mgm. compared to a mean of 66·8 mgm. for the rest of the year. Again the difference between these means is statistically significant, t value 2·12, $P = 0{\cdot}05$. Seven juvenile females collected late July to late October showed an average thymus weight of 2412 mgm. As in the males there was no evidence of a recrudescence of the bursa of Fabricius; its average weight was 45 mgm. in the adult females and 1311 mgm. in the juvenile females.

House Sparrows

Seasonal trends in the thymus weight and weight of both testes of adult male house sparrows are shown in Fig. 3. There is evidence of a thymus enlargement during June, July, and August though it is not evident in all birds collected during this period. The mean thymus weight of all birds

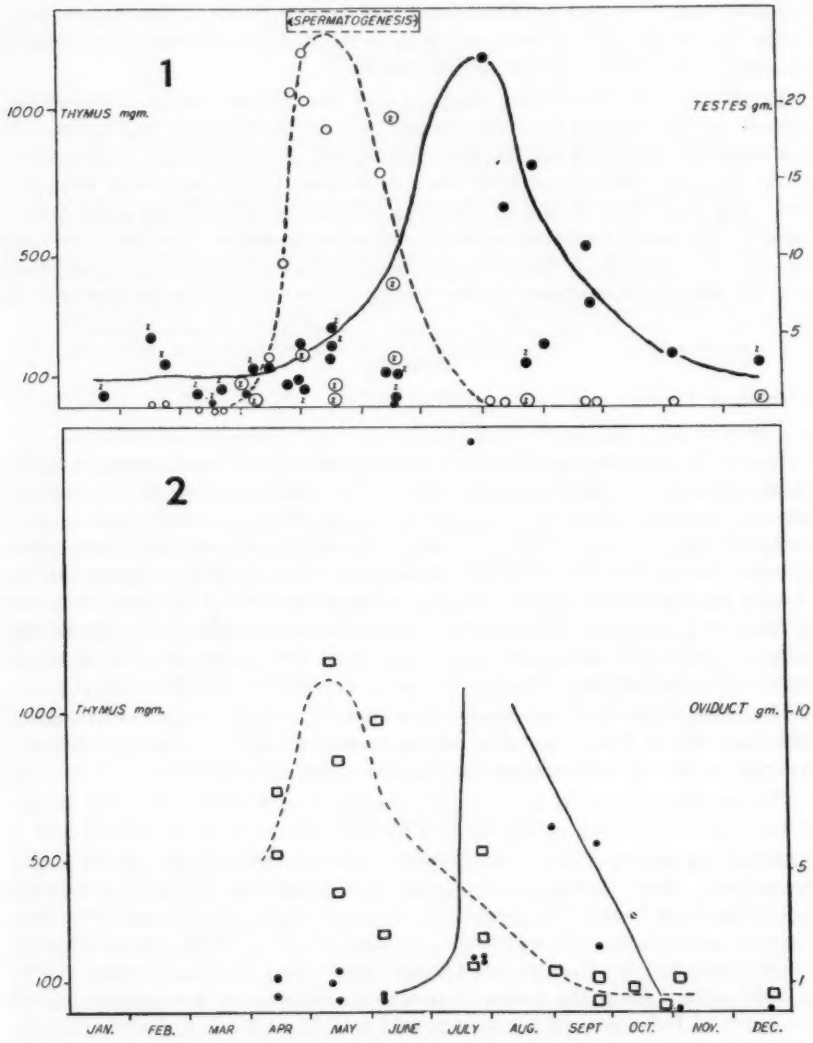

FIG. 1. Seasonal changes in thymus weight (continuous line, black circles) and weight of both testes (interrupted line, open circles) of adult male mallards.

FIG. 2. Seasonal changes in thymus weight (continuous line, black circles) and weight of oviduct (interrupted line, open squares) of adult female mallards.

examined during the period June 1 to August 31 is 11·5 mgm., mean thymus weight for those collected during the rest of the year is 6·1 mgm. Application of the *t* test indicates that this difference can be considered significant, *t* value 2·08, *P* = approximately 0·05. The average thymus weight of seven juvenile males collected July to December was 29 mgm. The bursa of Fabricius could not be found in any of the adult males, its average weight in the juvenile males was 31 mgm.

Weight curves for thymus and oviduct of adult females are shown in Fig. 4. A postbreeding season thymus enlargement is evident, involving the period July to September. Mean thymus weight for birds collected during this period was 36·7 mgm. and only 8·9 mgm. for the rest of the year. The difference of these means is highly significant, the *t* test gives a value of 6·67, *P* = 0·01. Juvenile females collected October to January had an average thymus weight of 31 mgm. No remains of the bursa were found in any of the adults while in the juvenile females it had an average weight of 19 mgm.

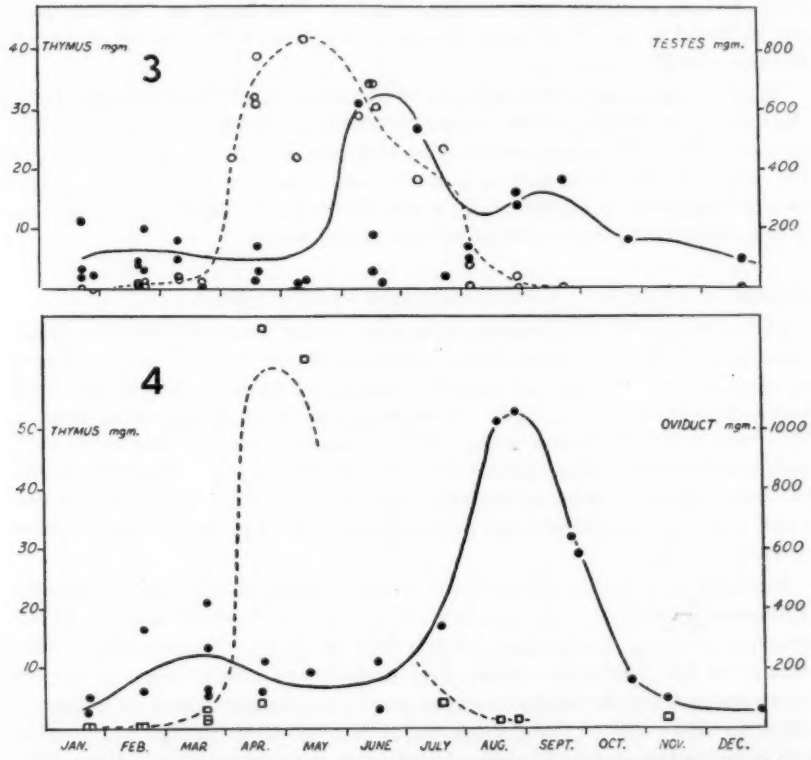

FIG. 3. Seasonal changes in thymus weight (continuous line, black circles) and weight of both testes (interrupted line, open circles) of adult male house sparrows.

FIG. 4. Seasonal changes in thymus weight (continuous line, black circles) and weight of oviduct (interrupted line, open squares) of adult female house sparrows.

Robins

Seasonal trends in the weight of the thymus, both testes, and the vas and seminal glomus of one side of adult male robins are shown in Fig. 5. Thymus weights of juvenile males are also shown in this figure. In this case the evidence of a thymus enlargement in the adults during June to September was so obvious that statistical tests of significance were deemed unnecessary. It is noteworthy that in this case the thymus enlargement overlapped the period of gonadal activity as indicated by testicular weight, spermatogenesis, and development of vas and glomus. A bursa of Fabricius could not be found in any of the adults; in eight juvenile males it had an average weight of 130 mgm.

Thymus weights of adult and juvenile female robins and oviduct weights of the adult females are shown graphically in Fig. 6. Again there is clear cut evidence of a thymus enlargement following the breeding season and beginning in July. The duration of the period of enlargement remains unknown as no adult females could be secured after August. The bursa of Fabricius could not be found in any of the adult females; its average weight in seven juvenile females was 217 mgm.

There is thus satisfactory evidence of an enlargement of the thymus during the summer in adults of both sexes in the mallard, house sparrow, and robin. In male robins the enlargement begins while the gonads are also enlarging; in the other two species as well as in the female robins the thymus enlargement does not begin till retrogression of the gonads has set in. The bursa of Fabricius is not enlarged in adults of these species at any season.

Evidence of a Summer Thymus Enlargement in Other Species

In the course of ornithological field work in the western Canadian Arctic, dissection of the thymus and bursa was carried out in a few representatives of other species. A male old squaw (*Clangula hyemalis*) killed July 21, 1953, had a thymus weight of 1 gm. A male pintail (*Anas acuta*) killed July 26, 1955, and a female greater scaup (*Nyroca marila*) obtained August 8, 1955, both had enlarged thymi estimated to weigh over 1 gm. Thymus weights for these species at other seasons are unknown, but there is little doubt that 1 gm. is well above the average thymus weight likely to be obtained at other times of the year.

Evidence of a thymus enlargement was also obtained in Lapland longspurs (*Calcarius lapponicus*). In a male shot July 27, 1953, which was still in full breeding plumage, no thymus tissue could be found. In two other males already in fall plumage, and therefore probably at a later stage of the sex cycle, and in a female the thymus lobes were as large as in two juvenile females killed the same day. The thymus tissue of these birds though not weighed was estimated as about 30 mgm. In an adult male horned lark (*Eremophila alpestris*) shot July 22, 1953, the thymus was about as large as in a juvenile shot the same day.

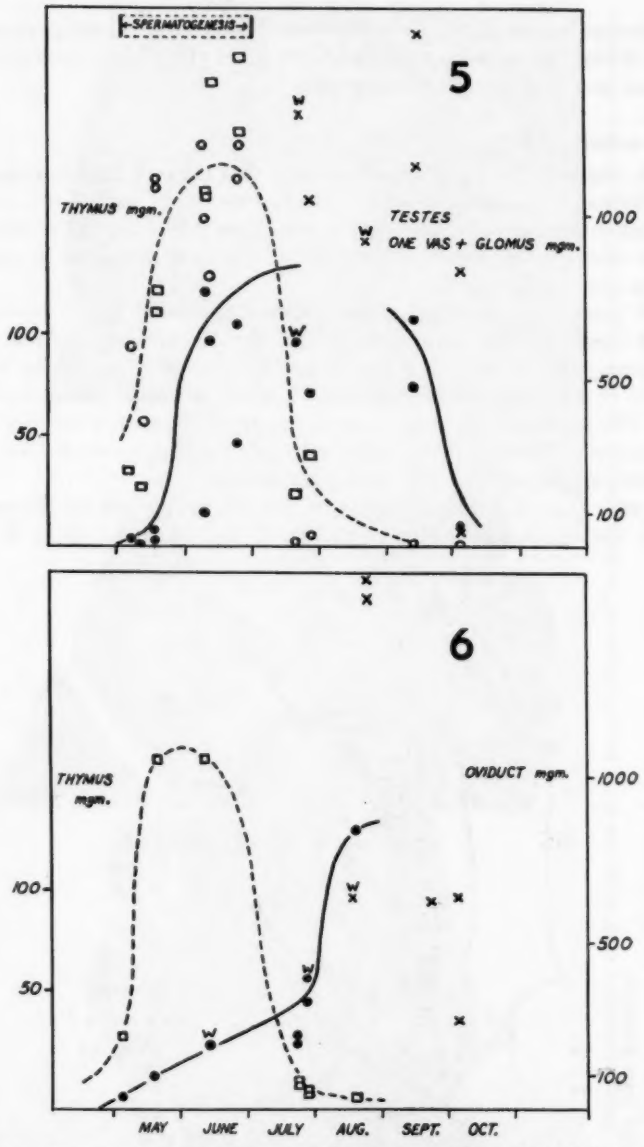

FIG. 5. Seasonal changes in thymus weight (continuous line, black circles), weight of both testes (interrupted line), and weight of one vas and glomus (open squares) of adult male robins. Thymus weights of juvenile males are also shown by "x". "W" indicates presence of cestodes in abdominal cavity of the specimen.

FIG. 6. Seasonal changes in thymus weight (continuous line, black circles) and oviduct weight (interrupted line, open squares) of adult female robins. Thymus weights of juveniles shown by "x". "W" indicates presence of cestodes in the abdominal cavity of the specimen.

It therefore seems highly probable that a considerable enlargement of the thymus during the summer is widespread in birds which have reached breeding condition, and possibly affects all species.

Gross Anatomy of the Avian Thymus

In the course of this work it became clear that the well known arrangement of the thymus in chickens, illustrated by Nonidez and Goodale (8), consisting of a number of lobes along the jugular vein from about the upper one-fifth of the neck down to the level of the thyroid gland, does not apply in any of the passerine species examined.

In all passerines examined, namely house sparrow, robin, horned lark, Lapland longspur, Bohemian waxwing (*Bombycilla garrula*), and rusty blackbird (*Euphagus carolinus*), the two largest lobes were found in the region of the angle of the jaw. The typical arrangement for these species is shown in Fig. 7. The presence of thymus tissue in the upper thorax is a variable feature. This pattern of thymus distribution was also found in a duck hawk (*Falco peregrinus*) and a pigeon hawk (*Falco columbarius*).

The other type of pattern typified by the chicken, where no thymus lobes occur in the uppermost region of the neck, was found to apply in species

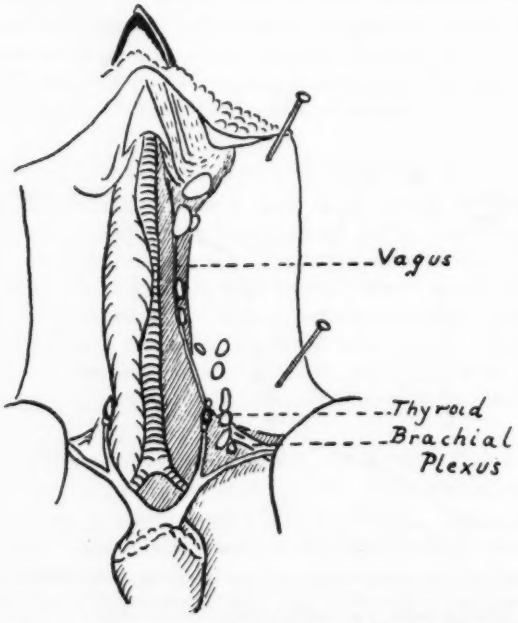

FIG. 7. Dissection of the thymus on the left side of the neck in an immature female house sparrow killed January 23, 1955. On the right a large jugular vein accompanies the vagus, on the left it is too small to be readily visible. The presence of thymus tissue below the level of the thyroid is unusual.

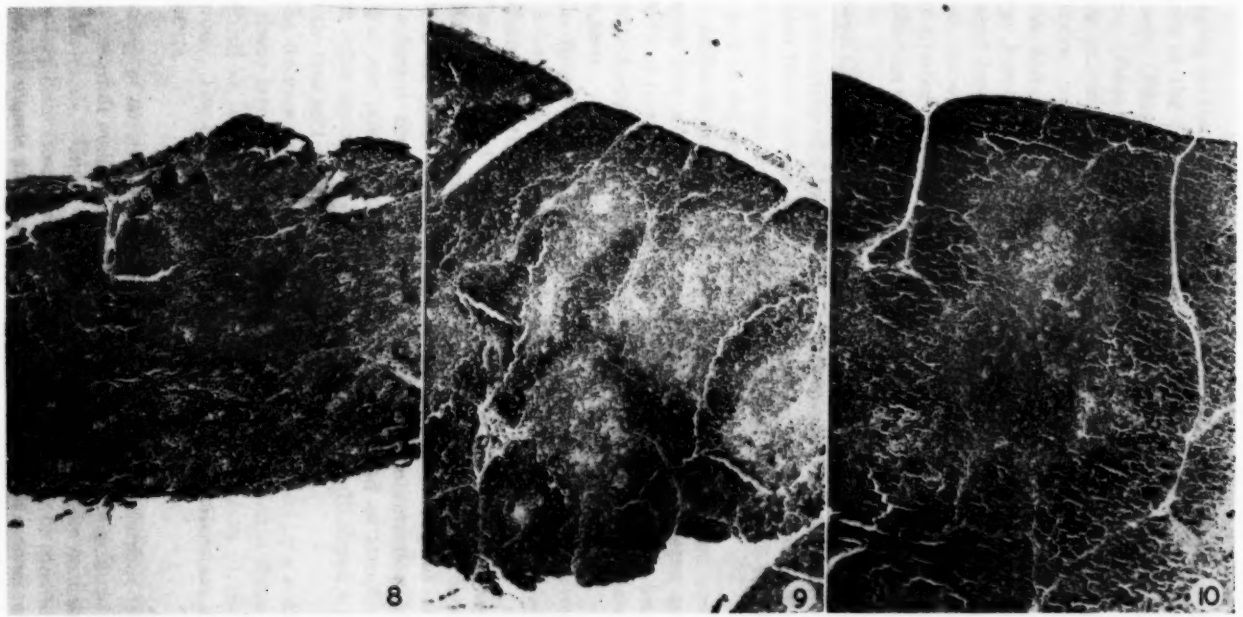

FIG. 8. Photomicrograph (× 50) of portion of the atrophic thymus of an adult female robin shot May 16, 1954. No division into cortex and medulla, little lobulation.

FIG. 9. Photomicrograph (× 50) of enlarged thymus of an adult female robin shot July 16, 1954. Lobulation well marked, a dark peripheral cortex and a paler more central medulla are evident.

FIG. 10. Photomicrograph (× 50) of uninvoluted thymus of an immature female robin shot August 21, 1954. Virtually indistinguishable from the adult thymus in recrudescence shown in Fig. 9.

belonging to the orders Galli, Laro Limicolae, Colymbi, and Anseres. Individuals of the following species of these four orders were examined; Galli: pheasant (*Phasianus colchicus*), spruce grouse (*Canachites canadensis*), ruffed grouse (*Bonasa umbellus*), sharp-tailed grouse (*Pedioecetes phasianellus*), willow ptarmigan (*Lagopus lagopus*), rock ptarmigan (*Lagopus mutus*), and European partridge (*Perdix perdix*). Laro Limicolae: Baird's sandpiper (*Erolia bairdi*), Hudsonian godwit (*Limosa haemastica*), and glaucous gull (*Larus hyperboreus*). Colymbi: yellow-billed loon (*Gavia adamsi*) and red-throated loon (*Gavia stellata*). Anseres: mallard, pintail, greater scaup, old squaw, snow goose (*Chen hyperborea*), blue goose (*Chen caerulescens*), and white-fronted goose (*Anser albifrons*). Possibly other patterns of thymus distribution may be found in orders of which no representatives were examined.

Seasonal Changes in Thymus Histology in the Mallard, House Sparrow, and Robin

Examination of a large number of thymus sections of adults and juveniles of these three species showed that the enlarged thymi of adult birds had the same microscopic structure as the thymi of juvenile birds in which no involution had as yet occurred.

In mallards and robins these thymi showed lobulation and a well defined division into a cortical and medullary zone. The cortex consisted almost entirely of lymphocytes; in the medulla lymphocytes were less concentrated, islands of epithelial cells were frequent, and occasional Hassal's corpuscles were seen.

The enlarged thymi of adult sparrows and of juvenile sparrows were similar but lobulation was never marked and no definite Hassal's corpuscles were encountered in the medulla.

The unenlarged thymi of adult birds of all three species were similar: lobulation was lost and it was not possible to recognize a cortex and medulla. Instead the tissue present contained all the elements found in the medulla of the enlarged thymi but with an increased concentration of lymphocytes. Typical low power appearance of an enlarged and an atrophic thymus of an adult robin are shown in Figs. 8 and 9, while Fig. 10 shows a low power view of a thymus section from a juvenile robin.

The thymus enlargement is therefore accompanied by a redifferentiation of the tissue into a cortical and a medullary zone, from a tissue which may be regarded as intermediate between these two elements of the non-involuted thymus. The enlargement obviously entails a proliferation of all the tissue elements.

Histology of the Atrophic Bursa Fabricii of Adult Mallards

In the adult mallards some remainder of the bursa was found in the majority of individuals. Under the microscope these threadlike structures, which had obviously been closed off from the cloaca, were seen to consist predominantly of smooth muscle and fibrous connective tissue with inconstant small islets of lymphocytes and remains of the epithelium lining the original, now occluded, lumen of the structure.

Delayed Involution of the Thymus and Bursa Fabricii in Birds Which Require More Than One Year to Reach Sexual Maturity

The great majority of birds reach full sexual maturity in the spring of the year succeeding the year of birth, i.e., when approximately one year old. This is the case with the three species under consideration here. However, many of the larger birds do not reach breeding condition until their second or even subsequent springs. It might be expected that in these species involution of the thymus and bursa is correspondingly delayed. This appears indeed to be true judging by some geese and a yellow-billed loon examined on S.W. Banks Island in 1953.

Blue goose.—Two immature birds collected on June 1 had enlarged thymi and bursae; the tissues were not weighed but were at least 1 gm. in weight each.

Lesser snow goose.—Three immature birds collected June 1 had enlarged thymi and bursae, each tissue (unweighed) weighing at least 1 gm. An adult examined the same day had a much smaller thymus and no bursa could be found. Three immature birds examined on July 13 all had enlarged thymi and bursae.

Yellow-billed loon.—A male which on plumage characters alone would have been considered an adult had a large thymus and bursa.

Attempts to produce a thymus enlargement out of season by various experimental procedures

1. *Effect of Castration on the Thymus and Bursa in Adult Mallards*

In view of the fact that thymus recrudescence in the species studied occurred after the annual postbreeding season regression of the gonads (though as noted above the thymus enlargement in male robins begins earlier), it seemed possible that thymus enlargement might be due to temporary cessation of an inhibitory influence exerted by gonadal hormones. An inhibitory effect of gonadal hormones or gonadotrophins on thymus development has been shown in certain mammals (1, 2) and an increase in thymus weight has been reported following castration in the rat (9).

If the thymus enlargement were due to decreased gonadal hormone secretion it should be possible by castration to bring about a thymus enlargement at times of the year when the thymus is normally small. Accordingly a number of male mallards were castrated under ether chloroform anesthesia and killed one month later when the weights of the thymi and bursae were recorded. Control birds were similarly anesthetized and sham operations were performed, in which each testis was simply exposed by an adbominal incision which was then closed again. The results of these experiments are shown in Table I. The castrates shown are only those in which macroscopic examination showed no testicular tissue at post mortem or where tissue which might represent regenerated testicular remains was shown by microscopic examination not to contain recognizable testicular elements.

TABLE I

EFFECT OF CASTRATION ON WEIGHT OF THYMUS AND BURSA IN MALLARDS

Operation performed one month before the birds were examined (N. fd. = not found)

Controls			Castrates		
Body wt., gm.	Thymus, mgm.	Bursa, mgm.	Body wt., gm.	Thymus, mgm.	Bursa, mgm.
898	121	23	860	112	7
900	142	51	1105	237	N. fd.
988	143	51	1025	1726	117
960	120	22	945	114	64
			1114	24	12
			1180	266	26
			1200	96	14
			900	75	21
			915	103	29
			1070	140	N. fd.

It is clear from these results of 10 complete castrations that the weights of the thymus and bursa were unaffected except in one bird where a thymus weight of 1726 mgm. and a rather high bursal weight of 117 mgm. were recorded. In view of the consistent lack of any evidence of thymus regeneration in the remaining nine birds the thymic enlargement seen in this one case cannot justifiably be attributed to the castration and is most probably an example of delayed involution of thymus and bursa, which normally is complete about the end of the first winter in the life of these birds. (The bird in question was examined in early March.)

2. *Effect of Unilateral Adrenalectomy on the Thymus and Bursa in Adult Male Mallards*

In mammals it is well known that adrenal corticoids can induce a rapid shrinking of the thymus and lymphoid tissue in general. This effect probably also occurs in birds since injections of adrenal cortical extracts in adult fowls can induce lymphopenia (11). Seasonal thymic enlargement might therefore be due to a temporary decrease of an inhibitory influence normally exerted by the adrenal cortex.

Accordingly one adrenal gland was completely excised under ether chloroform anesthesia in a number of adult mallard drakes. The birds were examined one month after the operation. The results of this procedure on thymus and bursal weight are shown in Table II. The weight of the remaining adrenal gland is also recorded and should be compared with an average adrenal weight of 119 mgm. in 26 normal male mallards.

The controls shown in Table I are also applicable here as the general operative procedure is similar to that used in the sham castrations.

TABLE II

EFFECT OF UNILATERAL ADRENALECTOMY ON WEIGHT OF THYMUS AND BURSA IN ADULT MALE MALLARDS

(N. fd. = not found)

Body wt., gm.	Thymus wt., mgm.	Bursa wt., mgm.	Remaining adrenal tissue, mgm.
885	95	N. fd.	85
930	25	N. fd.	60
890	37	N. fd.	117
835	126	20	91
1035	146	16	94

It is clear that removal of one adrenal did not increase the thymus or bursal weight in these five birds. While negative effects of complete adrenalectomy would be needed to exclude completely the possibility that the naturally occurring thymus enlargement might be due to decreased adrenal corticoid secretion, it seems unlikely that a naturally occurring postbreeding season reduction in adrenal cortical secretion would be greater than was achieved by removal of one adrenal gland. It will be noted that in one case there is no evidence of compensatory hypertrophy of the remaining adrenal, as it weighed about one-half the normal weight of both glands, in three others there has been about a 50% increase in the weight of the remaining gland, while in the fifth bird the remaining gland had approximately doubled its weight.

Discussion

The occurrence of a marked postbreeding season enlargement of the thymus, not accompanied by any recrudescence of the bursa, would appear to suggest that the thymus, at this period, performs some function that does not involve the whole lymphatic system, since it does not affect the lymphoid tissue of the bursa. The most striking bodily event taking place in birds after the breeding season is the annual molt. However, it is already known that thymectomized domestic fowl and pigeons undergo a normal molt (6, 7, 10). The thymus, at least in these two species, therefore does not play an essential role in the molting process. Neither is there in the three species studied a close relationship in time between onset of the thymic enlargement and the molt. The purpose of the observed enlargement is therefore still quite obscure.

As regards the mechanism by which the enlargement is brought about, the results of the castration experiments in mallards reported above and the almost simultaneous recrudescence of thymus and testes in male robins definitely rule out the possibility of attributing the thymic enlargement to a temporary decrease of sex hormone secretion from gonads temporarily exhausted by their great "spurt" of activity in the breeding season.

Although unilateral adrenalectomy in mallards did not bring about a thymic enlargement, a more marked decrease of adrenal cortical activity than was achieved by this procedure can still be considered as a possible explanation of the phenomenon.

A seasonal change in thyroid activity may be offered as an alternative explanation of thymus recrudescence. In man hyperthyroidism is very frequently associated with thymic enlargement (5). There is also considerable evidence that thyroid activity in birds is increased for some weeks preceding the onset of the annual molt, which in most species follows the breeding season (4). It may therefore be suggested that increased thyroid activity associated with the molt is responsible for the thymus enlargement which is observed at about the same time.

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THE STERIC FACTOR IN THE HYDROLYSIS OF β-1,4′-OLIGOGLUCOSIDES BY MYROTHECIUM CELLULASE[1]

By D. R. Whitaker

Abstract

A comparison of the rate constants and activation energies for the hydrolysis of cellobiose, cellotriose, cellotetraose, and cellopentaose by *Myrothecium* cellulase showed that while the rate constant was increased by a factor of about 450 as the degree of polymerization (D.P.) of the substrate was increased from two to five, the activation energy remained at about 12,000 cal. The results are interpreted, in terms of classical collision theory, as indicating that the increase in rate constant with D.P. is determined by an increase in the steric factor with D.P. Addition of a β-linked sorbityl group to an oligoglucoside increased the rate constant; the increase was less than that from addition of an anhydroglucose unit and, relative to the latter, diminished as the D.P. of the chain undergoing addition was increased. Exposing the enzyme to conditions favoring thermal or surface denaturation caused varying losses in enzymic activity towards the four oligoglucosides; wherever the loss in activity towards one oligoglucoside differed substantially from the loss in activity towards any other oligoglucoside, the greater loss was shown towards the substrate of lower D.P. The results are discussed.

Introduction

According to the present evidence (4, 6, 17), the cellulase of the mold *Myrothecium verrucaria* degrades the cellulose chain by more or less random cleavage of glucosidic linkages. A fully extended cellulose chain with a degree of polymerization (D.P.) of 2000, i.e. a chain containing 2000 anhydroglucose units, is about 10,000 Å long. Further almost all its glucosidic linkages are remote from the monosubstituted glucose units at the ends of the chain. As an indication of relative orders of magnitude, the hydrodynamic properties of *Myrothecium* cellulase correspond to those of a prolate ellipsoid with a length of about 200 Å (20). It might be expected therefore that cleavage by this enzyme of chains with D.P.'s in the range typical of native cellulose would be comparatively little influenced by "length effects", resulting from the variable lengths of the chains, or "end effects", resulting from the variable positions of the linkages with respect to the ends of the chains. The experimental evidence is incomplete but Greathouse has shown (4) that the viscosity-average D.P. of hydrocelluloses can vary from 120 to 1675 without significantly influencing the rate of enzymic degradation.

These considerations do not apply however to chains with degrees of polymerization as low as those of the soluble β-1,4′-oligoglucosides and, as reported previously from this laboratory (18, 19, 21), the enzymic hydrolysis of the oligoglucosides containing from two to six anhydroglucose units is influenced by the D.P. of the substrate in two respects. The first influence is on the rate of hydrolysis which increases sharply with the D.P. of the substrate up to, at least, a D.P. of five. The second is on the method of cleavage. In hydrolyzing cellotriose, the enzyme shows a substantial

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preference for cleaving the chains at a particular linkage; in this case, the glucosidic linkage adjacent to the non-reducing end of the chain. This tendency is still evident, though less marked, in the cleavage of cellotetraose, the preferred point of cleavage being the central linkage of the chain, and progressively less marked in the cleavage of cellopentaose and cellohexaose. The trend is, then, for the method of cleavage to become more random as the D.P. of the substrate increases.

In this paper, three types of evidence bearing on the nature of these influences are reported. The first is a comparison of the velocity constants and activation energies for the hydrolysis of the series of oligoglucosides from cellobiose to cellopentaose. The point at issue may be outlined as follows. Let the general term k_i denote the second order velocity constant for the hydrolysis of the oligoglucoside of the ith degree of polymerization. As in the following equation:

$$[1] \qquad k_i = A_i e^{-E_i/RT} ,$$

its magnitude at the absolute temperature T can be considered to be determined by two parameters: the Arrhenius activation energy, E_i, and the frequency factor, A_i (3, 5, 9). According to the classical collision theory of rate processes, A_i is determined by two other parameters:

$$[2] \qquad A_i = Z_i P_i$$

where Z_i is a collision number and P_i is a "steric" or "probability factor" (5, 9). The increase of k_i with i may thus originate, according to equation [1], from the activation energy decreasing or the frequency factor increasing as the D.P. of the substrate increases; if the latter, then according to equation [2], it may originate from the collision number or the steric factor increasing with D.P. These possibilities are considered in turn.

The second and third types of evidence are concerned respectively with the effects on the rate constant of modifying the substrates by converting the oligoglucosides to their sorbityl derivatives and with the effects on enzymic activity of modifying the enzyme by exposure to conditions favoring denaturation.

Materials and Methods

1. Materials

The enzyme was obtained from culture filtrates of the mold *Myrothecium verrucaria* and purified by methods previously described (17). The cellobiose was of reagent grade (Kerfoot's). Cellotriose, cellotetraose, and cellopentaose were isolated from an acetolyzate of cellulose by a procedure differing from that previously described (19) only in that the preliminary precipitation of the acetolyzate from cold methanol was omitted.

Prior to addition, the enzyme and substrate were dissolved in acetic acid – sodium acetate – sodium chloride buffers of pH 5.0 and ionic strength 0.05. Their composition was chosen so that the buffering capacity, while sufficient

to maintain a constant pH during the hydrolyses, was not high enough to interfere with the method of analysis for reducing sugar. They were 0.01 *M* with respect to sodium acetate and 0.04 *M* with respect to sodium chloride. The acetic acid concentration of each buffer was such as to give a pH of 5.0 at the temperature for which the buffer was intended. Standardizations were made against a United States Bureau of Standards potassium phthalate buffer.

2. Analytical Method

The formation of reducing sugars was measured by Nelson's colorimetric modification of the micro-Somogyi method (10). The period for copper reduction was increased to 30 min. and after color development the volume was made up to 25 ml. The reduction in optical density, as measured in 1 cm. cuvettes by a Spekker photoelectric colorimeter with an Ilford 604 filter, will be referred to hereafter as the "Nelson value".

Equimolar solutions of cellobiose, cellotriose, cellotetraose, and cellopentaose in acetate – sodium chloride buffer gave approximately the same Nelson value. These values were appreciably higher than those of equivalent solutions of glucose (United States Bureau of Standards reagent), the ratio of the Nelson values for cellobiose to those for glucose being approximately 1.12. Glucose is a comparatively minor initial hydrolysis product of cellotetraose and cellopentaose (19) and, in calculating velocity constants for the hydrolysis of these substrates, no correction was made for the lower Nelson value of glucose, the ratio S_0/S_t, where S_0 is the substrate's initial molar concentration and S_t is its concentration after hydrolysis for t minutes, being taken as equal to $D_0/2\ D_0 - D_t$, where D_0 is the initial Nelson value of the substrate solution and D_t is the Nelson value of the hydrolyzate at time t. For cellotriose, S_0/S_t was taken as equal to $D_0/[D_0 - 1.12\ (D_t - D_0)]$; for cellobiose, S_0/S_t was taken as equal to $D_0/[D_0 - 1.27\ (D_t - D_0)]$. The corrections in these last two expressions are equivalent to correcting D_t for the lower Nelson value of glucose in the hydrolyzate and, for convenience, will be indicated as $D_0/(2\ D_0 - D_t^*)$. The condition is implied in these expressions that the increase in Nelson value is entirely due to hydrolysis of the original substrate although all the substrates but cellobiose can give rise to hydrolysis products which can undergo further hydrolysis. However, these products are hydrolyzed at considerably slower rates than the original substrate and for the present conditions it can be shown that the contribution of secondary hydrolysis to D_t becomes appreciable only when the ratio D_t/D_0 has attained a fairly high value (>1.4)[2].

3. Determination of Velocity Constants and Activation Energies

The conditions for hydrolysis were as follows: an enzyme concentration of 20 μgm. protein/ml., a substrate concentration of *ca.* 0.0005 *M*, and temperatures of 9.82, 19.25, 29.41, and 38.90° C. The enzyme concentration was a compromise between the high enzyme concentration desirable for avoiding

[2] *The method of calculation is given in (19); the data necessary for the calculation is in Table II and (19).*

too slow a rate of hydrolysis of cellobiose and the low enzyme concentration desirable for avoiding too fast a rate of hydrolysis of cellopentaose. Previous data on the hydrolysis of these sugars (19) indicated that all reactions were likely to be second order only at very low substrate concentrations (<0.003 M). The substrate concentration chosen was considered to be about the lowest concentration which could be analyzed with a precision of about one per cent by the Nelson method.

In most cases the reaction vessels were Thunberg tubes. One milliliter of enzyme solution and 9 ml. of substrate solution were equilibrated to temperature, the former in the side arm, the latter in the main body of the tube, and then mixed by inverting the tube. At various time intervals, 1 ml. samples were removed and added to Folin-Wu tubes containing the Nelson reagents. The hydrolysis of cellopentaose was also carried out by a second method which enabled the reaction to be followed over shorter time intervals. One milliliter samples of substrate solution were added to a number of Folin-Wu tubes and, after temperature equilibration, the reaction was started by the addition of 0.1 ml. of enzyme solution to each tube. The reaction was stopped by the addition of 1 ml. of the copper reagents.

Velocity constants were estimated from the slope of the line obtained, as shown in Fig. 1, by plotting $\ln D_0/(2\,D_0 - D_t)$ or $\ln D_0/(2\,D_0 - D_t^*)$ against t.

Activation energies were estimated from a least squares regression of $\ln t$ on $1/T$ (5) where t is the time in minutes required at the absolute temperature T for the Nelson value to increase by a specified amount. Except for cellobiose, for which a somewhat lower increase has to be accepted, the increase specified was 40% of the initial Nelson value.

4. Preparation and Hydrolysis of Sorbityl Derivatives of the Oligoglucosides

Approximately one gram of each oligoglucoside was catalytically hydrogenated under the conditions used by Levene and Kuna (7) for reducing cellobiose to 4-β-glucosidosorbitol. The Raney nickel catalyst was prepared by the method of Pavlic and Adkins (12). After hydrogenation for 48 hr. at a pressure of from 3000 to 4000 p.s.i., the products showed no reducing power towards the Nelson reagents. The reduced sugars were adsorbed on charcoal columns (16) and the columns washed with several liters of distilled water, followed by increasing concentrations of aqueous ethanol. Reduced cellobiose was eluted with 8% (v/v) ethanol, reduced cellotriose with 15% ethanol, reduced cellotetraose with 25% ethanol, and reduced cellopentaose with 35% ethanol. The progress of elution was followed by spot tests on the eluate with a solution of anthrone in concentrated sulphuric acid. The eluates were concentrated under reduced pressure to sirups which, on trituration with absolute ethanol and drying in a vacuum oven, filled the containing vessel with a voluminous, friable solid. The weights were from 60 to 80% of the weight of the starting products.

Paper chromatograms of the products showed only one component when sprayed with alkaline silver nitrate (15). With the organic phase of a mixture

of isoamyl alcohol, pyridine, and water (1 : 1 : 1 v/v) as developing solvent, the products migrated at essentially the same rate as their parent sugars. With phenol–water (4 : 1 v/v), a solvent used by Parikh, Parikh, and Godbole (11) for separating sorbitol from glucose, the derivatives moved at slightly higher rates than their parent sugars. Paper chromatograms of acid hydrolyzed samples showed two components coinciding in position with glucose and sorbitol.

The conditions of enzymic hydrolysis were the same as those of the free sugars. In the Nelson analyses of the hydrolyzates, the volume after color development was made up to 12.5 ml. The substrates were appreciably hydrated and, in the calculation of the velocity constants for their enzymic hydrolysis, the initial substrate concentration was taken as the concentration estimated from Nelson analysis after acid hydrolysis (19).

5. Assay of Enzymic Activity in Denaturation Experiments

In these experiments, samples of a stock solution of enzyme in acetate–sodium chloride buffer were treated as described later (Table V). The activity of each sample was then measured relative to that of the stock solution from the increase in Nelson value after incubation with substrate.

To assay enzyme activity, 0.5 ml. of diluted enzyme solution was added to a Folin-Wu tube containing 0.5 ml. of 0.001 *M* substrate solution and incubated at 38.9° C. for periods which varied with the substrate as follows: cellobiose, 5 hr.; cellotriose, 30 min.; cellotetraose, 15 min.; cellopentaose, 6 min. The reactions were then stopped by the addition of 1 ml. of the Nelson copper reagent. The concentration of the diluted enzyme solution varied with the substrate as follows (concentration as μgm. protein/0.5 ml.):

Treated enzyme—cellobiose, 40 μgm.; cellotriose, 20 μgm.; cellotetraose, 10 μgm.; cellopentaose, 5 μgm.

Stock solution—in each assay, 100%, 75%, 50%, and 25% of the concentration of treated enzyme.

All assays were in duplicate. Solutions of substrate without enzyme showed no change in Nelson value during incubation. At the highest enzyme concentration in the assay, solutions of enzyme without substrate had Nelson values less than 0.01.

Results and Discussion

1. Hydrolysis of Oligoglucosides

Some representative data are given in Fig. 1 and Table I as evidence that the reactions were approximately second order under the conditions chosen for comparing velocity constants and activation energies. If the reactions are second order, i.e. if $-dS/dt = kSX$ where k is a constant, S is the molar substrate concentration at time t, and X is the molar enzyme concentration, then

[3] $$\ln \frac{S_0}{S} = kXt$$

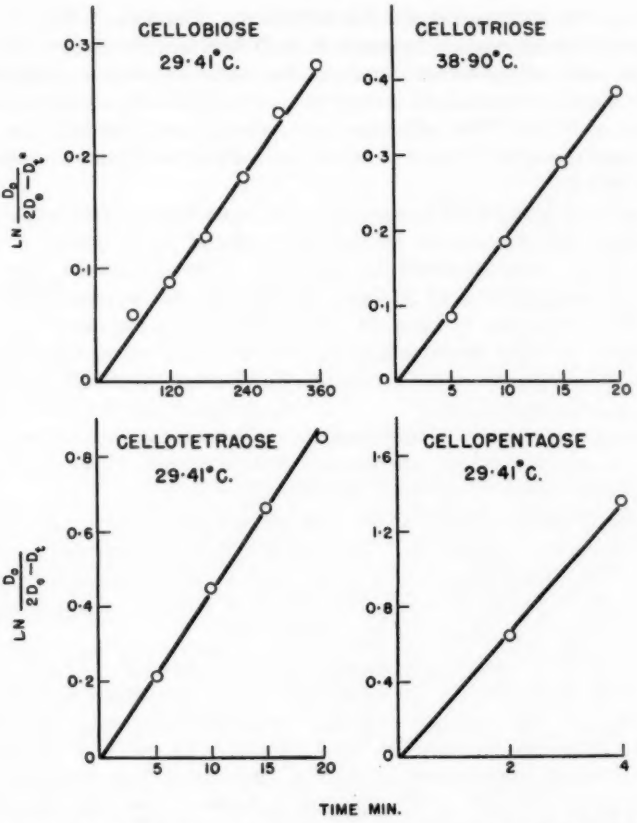

FIG. 1. Data for estimation of velocity constants for enzymic hydrolysis of oligoglucosides.

TABLE I

ENZYMIC HYDROLYSIS OF β-1,4'-OLIGOGLUCOSIDES: EFFECTS OF ENZYME CONCENTRATION (X)*

Substrate	Temp., °C.	X, μgm. protein/ml.	t, min.	$\ln \frac{D_0}{2D_0 - D_t}$
Cellobiose	38.9	20	291	0.44
		40	150	0.44
Cellopentaose	29.4	10	5.5	0.63
		20	2.5	0.63
Cellotriose	38.9	10	30	0.26
		20	30	0.54
Cellotetraose	38.9	10	10	0.45
		20	10	0.88

* *Substrate concentration: 0.0005 M.*

where S_0 is the initial substrate concentration. The data in Fig. 1 illustrate the required linear relation between ln S_0/S and t. The data in Table I for cellobiose and cellopentaose illustrate the required inverse proportionality between X and t when ln S_0/S is constant (as in measuring activation energies). The data in Table I for cellotriose and cellotetraose illustrate the required direct proportionality between X and ln S_0/S when t is constant (as in assaying enzyme activity).

If rates of hydrolysis are compared at the same enzyme concentration, then the product kX of equation [3] may be replaced by K where K has the dimensions of a first order velocity constant. The estimated values of K for an enzyme concentration of 20 μgm. protein/ml. and temperatures of 19.2° and 29.4° C. are given in Table II. Each estimate is the mean value of two determinations. The deviations of the individual estimates from their mean value were less than seven per cent of the mean value for all velocity constants but that of cellobiose at 19.2° C. For this estimate, the deviation was 11%. The velocity constants for cellotetraose and cellopentaose may be low estimates as they are calculated from uncorrected Nelson values. However the corrections would be small, particularly for cellopentaose (19).

The general trend is similar to that determined previously for a less comparable set of reaction conditions. The rate of hydrolysis increases very sharply as the D.P. increases from two to three, more slowly for succeeding increases in D.P. A net effect of increasing the D.P. from two to five is to multiply the velocity constant by a factor of from 400 to 450.

The estimated activation energies are given in Table III. The mean values are the total regressions for the combined data. One estimate of the activation energy for cellopentaose hydrolysis and both estimates for cellobiose hydrolysis are based on data for three temperatures only: in the first case, the hydrolysis of cellopentaose at 38.9° C. was too fast to estimate t by the usual procedure (time for a 40% increase in Nelson value—less than 90 sec.); in the second case, the hydrolysis of cellobiose at 9.82° C. was too slow for accurate measurement of the change in Nelson value. The estimation of activation energies from ln t rather than from ln K has the advantage, in

TABLE II

ENZYMIC HYDROLYSIS OF β-1,4′-OLIGOGLUCOSIDES: VELOCITY CONSTANTS*

Substrate	Temperature, 19.2° C.		Temperature, 29.4° C.	
	K, min.$^{-1}$	K/K_{g-1}†	K, min.$^{-1}$	K/K_{g-1}
Cellobiose	3.3×10^{-4}	—	7.0×10^{-4}	—
Cellotriose	5.4×10^{-3}	16	9.9×10^{-3}	14
Cellotetraose	2.5×10^{-2}	4.7	5.0×10^{-2}	5.0
Cellopentaose	1.32×10^{-1}	5.3	3.1×10^{-1}	6.2

* *Enzyme concentration: 20 μgm. protein/ml.*
† *K_{g-1} is the velocity constant of the sugar of next lower D.P.*

TABLE III

ENZYMIC HYDROLYSIS OF β-1,4'-OLIGOGLUCOSIDES: ACTIVATION ENERGIES

Substrate	Trial	Activation energy,* calories/mole
Cellobiose	1	12,700 ± 800
	2	12,400 ± 1100
	Mean	12,500 ± 600
Cellotriose	1	12,000 ± 210
	2	12,100 ± 190
	Mean	12,000 ± 150
Cellotetraose	1	12,200 ± 800
	2	11,900 ± 400
	3	12,100 ± 900
	Mean	12,100 ± 300
Cellopentaose	1	12,200 ± 600
	2	12,500 ± 900
	Mean	12,400 ± 700

principle, that t requires no correction for any differences between the Nelson equivalent of the substrate and its hydrolysis products. However essentially the same mean values were obtained by estimating E from K. Plots of ln t against $1/T$ showed no evidence of any systematic departure from linearity.

The data show no evidence of differences in activation energy being the determining factor in increase in velocity constant with D.P. The difference in activation energy necessary to account for the difference between the velocity constants for cellobiose and cellopentaose at 29.4° C. is about 3800 cal. This difference is about six times the standard error of the difference between the estimated activation energies for the hydrolysis of these substrates.

It is concluded that, in terms of the parameters of equation [1], the increase in the velocity constant is determined mainly by an increase in the frequency factor. The logarithm of the frequency factor is proportional to the entropy of activation (5) and possibly the most general interpretation of the trend is that the entropy of activation increases with the D.P. of the substrate. The interpretation implied in equation [2] may be less general—collision frequencies do not figure explicitly in the analysis of rate processes by transition state theory (3)—but, for the present purposes, it will be assumed. Of the two parameters in equation [2], the collision number, Z_i, is unlikely to vary significantly with i, at least insofar as it is determined by the properties of the substrate molecules alone. Increasing the D.P. of a chain increases the effective collision diameter but decreases the translational velocity; the two effects tend to balance and, although a large increase in D.P. can have secondary effects which reduce the collision number, a small increase in D.P. is unlikely to result in a large increase in Z (1, 9). Assuming for the present, as it will be discussed later, that the enzyme responsible for hydrolysis does not vary with i, it follows that the parameter of equation [2] which must increase with i is the steric factor, P.

According to this analysis, the hydrolysis of cellopentaose has the largest steric factor in the series. Its order of magnitude may be roughly estimated as follows. 1. The value of E in equation [1] is about 12,000 cal. 2. The molecular weight of the enzyme has been estimated to be about 63,000 (18). This estimate gives X in equation [3] a maximum value of 3.1×10^{-7} M. The corresponding minimum value of k at 29.4° C., from the value of K in Table II, is about 1.6×10^{4} liters/mole/sec. (3). The remaining factor, Z, may be calculated, if only to its order of magnitude, from the collision formula given by Kinetic theory (9):

$$Z = \frac{N_0}{1000} (r_E + r_S)^2 \left[8 \pi RT \left(\frac{1}{M_E} + \frac{1}{M_S} \right) \right]^{\frac{1}{2}}$$

where N_0 is Avogadro's number, r_E and r_S are the respective collision radii of the enzyme and substrate molecules, and M_E and M_S are the respective molecular weights of enzyme and substrate. If the collision radii are taken as roughly equal to the radii of the spheres whose volumes are equal respectively to the volumes of the enzyme and substrate molecule, then $r_E \approx 30 \times 10^{-8}$ cm. and $r_S \approx 5 \times 10^{-8}$ cm. Accordingly, $Z \approx 1.9 \times 10^{12}$. On these approximations, $P \approx 3$, i.e., its order of magnitude is unity. In contrast, values of P as high as 10^8 have been reported for the acid-catalyzed hydrolysis of oligoglucosides (9).

On this estimate, the steric factors for the hydrolysis of this series of substrates range from about 3 for cellopentaose down to about 0.01 for cellobiose. Both the calculation and, in its present application, the theoretical basis of the calculation are too approximate for this range of values to put much restriction on the possible origins of the variation of the steric factor with D.P. However, from the nature of the problem, several of the main causes (9) of a reaction having a higher or lower rate than that predicted by the simple collision theory are unlikely. The following are suggested as two possible, though not necessarily independent, causes:

1. The required orientation of the substrate with respect to the active centers of the enzyme, i.e. the orientation necessary if encounter is to be followed by reaction, becomes less critical as the D.P. of the substrate increases.

2. The attractive forces which determine the formation of the enzyme–substrate complex increase with the D.P. of the substrate. The list is not exhaustive but will suffice as a background for the discussions in the following sections.

2. Hydrolysis of Sugar Alcohols

The estimated velocity constants for the hydrolysis of the sugar alcohols at 38.9° C. are given in Table IV. The ratio, K/K_{g-1}, where K is the velocity constant for the hydrolysis of the alcohol and K_{g-1} is the velocity constant for hydrolysis of the oligoglucoside containing one less glucosidic linkage, gives a measure of the effects on the rate of hydrolysis of adding a sorbityl group to an oligoglucoside chain. A comparison of these rates with those in Table II shows that while the addition of a sorbityl group increases the rate constant,

TABLE IV

ENZYMIC HYDROLYSIS OF SUGAR ALCOHOLS : VELOCITY CONSTANTS*

Substrate	K, min.$^{-1}$	K/K_{g-1}†	R‡
Sorbityl -β-glucoside	2.7×10^{-4}	—	—
Sorbityl -β-cellobioside	1.3×10^{-2}	9.3	0.62
Sorbityl -β-cellotrioside	2.9×10^{-2}	1.5	0.31
Sorbityl -β-cellotetraoside	9.8×10^{-2}	1.15	0.20

** Enzyme concentration, 20 μgm. enzyme protein/ml., temperature, 38.90° C.*

† K_{g-1} is the rate constant, at the same temperature, of the oligoglucoside containing one less glucosidic linkage than the substrate.

‡ R is the ratio of the k/k_{g-1} values in Table IV to the corresponding values in Table II.

the increase is invariably less than that for addition of an anhydroglucose unit, and in relation to the latter (R values in Table IV), falls off rapidly as the D.P. of the sugar undergoing addition is increased.

It might be inferred from these results that the influences determining the increase in rate constant with D.P. vary with the length of the chain undergoing addition. As the conversion of glucose to sorbitol entails cleavage of the glucopyranose ring, the structural changes responsible for the increase in rate constant from adding a sorbityl group to a chain may include little more than the replacement of the potential aldehyde group of the chain by a glucosidic linkage. Nevertheless, when the chain is cellobiose, their effects on the rate constant are comparable to those of an added anhydroglucose unit. One interpretation of the trend shown by the R values is that they reflect the diminution with increasing D.P. of the influence of an end effect on the rate of hydrolysis. A similarly diminishing influence of end effects is reflected by the decreasing selectivity as the D.P. increases, in the method of cleavage of the oligoglucosides. On this interpretation, the R value is greatest when cellobiose undergoes addition because the hydrolysis of cellobiose is most subject to the influence of end effects and the addition of either a sorbityl or an anhydroglucose unit gives a linkage, flanked by anhydroglucose units, which is not immediately adjacent to a terminal reducing group. The R values cannot be expected to reflect any influence associated with the proximity of a linkage to the non-reducing end of the chain. The method of cleavage of cellotetraose suggests that there is such an influence although, judging by the method of cleavage of cellotriose, less than that from the reducing end. In some respects, end effects might be considered as a consequence of the linearity of the chains. Another property of the oligoglucosides which may have some bearing on these trends is that a chain of β-linked sugars has a twofold screw axis and thus, from any aspect, adjacent anhydroglucose units present different arrays of functional groups. Influences associated with the number of combinations of arrays which a chain presents may thus vary differently with D.P. than influences associated with the number of linkages.

3. Denaturation Experiments

The treatments applied in these experiments and their effects on enzymic activity are given in Table V. The thermostats were water baths and, during the period of immersion in them, the flasks containing the enzyme solutions were given a gentle rotary shaking to increase the rate of heat transfer. However, in treatments involving brief exposures, the temperature attained by the enzyme solution was probably appreciably less than the temperature of the thermostat. The degree of foaming in treatment C varied considerably but was greatest for the solutions exposed to 70° C. None of the treated solutions showed any evidence of turbidity or precipitation. The residual activities given in the table are the mean values of two determinations. The maximum deviation of the individual estimates from their mean corresponded to a maximum deviation of about four per cent in the estimated activity; the average deviation was about two per cent.

The results show firstly that a pretreatment which results in a certain loss of enzymic activity towards one oligoglucoside may result in a significantly

TABLE V

EFFECT OF VARIOUS TREATMENTS ON ENZYME ACTIVITY

Temp., ° C.	Exposure time	Residual activity (%) towards			
		Cellobiose	Cellotriose	Cellotetraose	Cellopentaose
Treatment A: 16 ml. of enzyme solution (200 μgm. protein/ml.) added to 50 ml. volumetric flask in thermostat at 80° C. Samples withdrawn at times specified and diluted in cold buffer					
—	1 min.	7	12	50	—
—	2 min.	0	0	25	—
—	8 min.	0	1	17	—
—	16 min.	0	0	0	—
Treatment B: 5 ml. samples of enzyme solution (240 μgm. protein/ml.) added to 25 ml. volumetric flasks in thermostats at temperatures specified. Flasks withdrawn after 5.0 min. and cooled in ice bath					
46.2	—	100	100	100	100
53.0	—	87	87	85	102
58.0	—	46	48	45	98
65.1	—	4	3	21	90
69.6	—	1	0	15	75
75.6	—	0	0	10	19
80.7	—	0	1	5	13
Treatment C: 4 ml. samples of enzyme solution (200 μgm. protein/ml.) added to 10 ml. volumetric flasks in thermostat. Flasks removed at times indicated. Cold buffer then blown in to promote foaming					
60.1	30 sec.	97	96	94	96
60.2	60 sec.	67	65	69	75
70.1	30 sec.	10	27	50	82
70.4	60 sec.	1	5	49	81
79.3	30 sec.	3	37	45	64
79.7	60 sec.	2	18	30	32

different loss in activity towards another; secondly, that wherever such differences result, the greater loss is shown towards the substrate of lower D.P.

If the data in Table V is interpreted on the hypothesis that denaturation is an "all or none" process, the simplest interpretation is that each substrate is hydrolyzed by a separate enzyme. As discussed in a recent review by Putnam (13), the effects of denaturation on the physical properties of proteins throw considerable doubt on the validity of the "all or none" hypothesis. However, as it is not uncommonly assumed that evidence of such differential effects is proof of enzymic heterogeneity, it is of interest to assume the hypothesis and consider its consequences. The simplest interpretation will be adopted; otherwise it is necessary to postulate more than four enzymes. Then, on the "all or none" hypothesis it is necessary to assume the following.

1. The individual enzymes have a unique degree of specificity with respect to the D.P. of the substrate. For at least three of them, the specificity may be absolute with respect to either the number of glycosidic linkages or the number of anhydroglucose units in the chain but not both, unless sorbityl derivatives are hydrolyzed by other enzymes.

2. Although different, the enzymes all interact with their substrates in such a manner that the activation energies for the reactions are approximately the same.

3. The individual enzymes have similar physical properties as the enzyme preparation gives only one peak in the ultracentrifuge and on electrophoresis at three pH's and shows no evidence of heterogeneity on diffusion (17, 20).

4. Each enzyme has a molar ratio in the enzyme preparation and a steric factor associated with its action which together are such as to give the observed increase in rate constant with D.P. If the molar ratios are approximately the same then, despite 1, the lower the D.P. of the substrate, the less is reaction favored by the properties of the enzyme. If the steric factors are approximately the same, the relative molar ratios are given by the ratios of the velocity constants, e.g. the molar ratio of cellobiase is about 1/500th that of cellopentaase. However despite such ratios, the complex is not fractionable by at least three fractionation procedures (17).

5. Any trends shown with respect to D.P. by the effects of sorbityl addition, by differential losses in activity from exposing the enzyme to conditions favoring thermal or surface denaturation, or by the method of chain degradation are due to coincidence. The "all or none" hypothesis thus not only requires the postulation of several enzymes but requires that the enzymes and the enzyme preparation satisfy a rather improbable set of conditions.

On the alternative view (13)—that there are degrees of denaturation—these difficulties do not arise. The differential effects can then be interpreted as simply indicating that the enzyme, like the substrate, can undergo structural changes whose effects on enzymic activity vary with the D.P. of the substrate. The trend shown by the data in the previous sections suggests that the lower the D.P. of the substrate, the less is activity toward it favored by the properties of the enzyme; the trend shown by the differential effects of denaturation

suggests that the lower the D.P. of the substrate, the less is activity toward it favored by certain changes in the properties of the enzyme. The two trends thus supplement each other.

These differential effects can be interpreted as indicating that the influence of certain properties of the enzyme, like those of the substrate discussed in the preceding section, becomes increasingly critical as the D.P. of the substrate decreases. However, such trends could be accommodated by several different hypotheses, emphasizing, to differing degrees, the variation in attractive forces, repulsive forces, or orientation factors with D.P. Other types of evidence are necessary to resolve them and, for the present, the conclusions drawn from the results in these two sections are put forward as simply extensions of the conclusion that the increase in rate of hydrolysis with D.P. is determined mainly by the steric factors for the reactions.

Apart from their bearing on the problem just considered, the results in Table V are also of interest for comparison with other reports on the thermostability of this enzyme. Saunders, Siu, and Genest (14) found that the activity of culture filtrates of the mold, as assayed from the amount of soluble reducing sugar formed from cellulose, showed an appreciable loss after the filtrate was heated for 10 min. at 50° C. and complete loss after it was heated for 10 min. at 70° C. On the other hand, Marsh, Bollenbacher, Butler, and Guthrie (8) found that culture filtrates could be heated for 30 min. at 95° C. and still retain appreciable activity as assayed by ability to reduce the viscosity of solutions of carboxymethylcellulose and by a test, apparently for ability to damage the primary wall of cotton fibers, which defines what they refer to as "S-factor" activity. A similar order of thermostability is indicated by the data of Kooiman, Roelofsen, and Sweeris (6) for a crude enzyme preparation obtained by salting out the protein from culture filtrates of another strain of the mold. In this case, the property studied was the ability of the enzyme to form soluble hydrolysis products which could be detected by paper chromatography. Various statements in their paper indicate that the enzyme preparation had a high cellobiase activity. This activity was largely destroyed by boiling the enzyme solution for one minute. However activity towards a large group of other substrates, ranging from cellotriose to untreated cellulose from several sources, was still appreciable after the enzyme solution was boiled for three minutes.

The varying orders of thermostability indicated by these results and those in Table V probably reflect, in part at least, differences in the test conditions, e.g. in the pH, ionic strength, and protein concentration of the enzyme solution. Another variable is the composition of non-protein metabolic products in the culture filtrate. These include products which form complexes with proteins and evidence has been obtained recently in this laboratory which suggests that these products contribute to the thermostability of crude enzyme preparations. Differential effects as in Table V might also be of some importance as, for example, a viscometric assay, which largely measures the ability of an enzyme to cleave long chains, might be expected to be less sensitive to such effects

than an assay based on analysis for soluble reducing sugars. However differential effects would not account for the differing orders of thermostability indicated by the data of Table V and that of Kooiman *et al.*

The results in Table V also emphasize the importance of avoiding conditions which favor denaturation in the preparation, purification, and assay of cellulolytic enzymes. Gilligan and Reese (2) have recently claimed to have fractionated *Myrothecium* cellulase, by paper chromatography and other adsorption methods, into several different components. Their claim is based on the lack of direct correlation between the activities of their fractions towards carboxymethylcellulose, as measured by a viscometric assay with an incubation period of one hr. at 50° C., and their activities towards cellulose, as measured by a reducing sugar assay with an incubation period of two hours at 50° C. The incubation conditions used in their assays suggests that the properties under assay included the thermostability as well as the cellulase activity of their fractions. If so, a lack of correlation between assays can be interpreted in other ways.

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A STUDY OF THE EFFECT OF ANTIHISTAMINES ON THE METABOLISM OF HISTAMINE IN THE INTACT RAT[1]

By M. Goldner[2] and L. P. Bouthillier

Abstract

The effect of the antihistamines Neohetramine and Neo-Antergan on the catabolism of histamine-β-C^{14} in the intact rat was investigated. Our results, obtained by the application of paper chromatographic and isotope dilution techniques, indicate that the urinary pattern of imidazole compounds is not altered by these antihistaminic substances.

Introduction

The catabolic pathway followed by histamine in intact animals has been studied by a number of authors. It has been found that in the rat, neither the imidazole ring of histamine (6) nor the carbon side chain of this molecule (2) undergoes scission to any significant extent. The only product of histamine oxidation identified in the urine is imidazoleacetic acid (2, 8). Since imidazoleacetic acid is not catabolized further in the intact rat (3, 8), it follows that this metabolite remains the end product of histamine degradation. It has been shown, however, that imidazoleacetic acid is partly excreted in the form of a detoxication product, partially characterized as imidazoleacetic acid riboside (5, 8).

In the course of our work on histamine catabolism, we became interested in studying the effect of some antihistaminic substances on the metabolism of histamine-β-C^{14} administered to rats. The results of this investigation are hereinafter presented.

Experimental

Administration of Labeled Histamine and Antihistaminic Substances

One milliliter solutions of 9·5 and 19·0 mgm. of histamine-β-C^{14} (specific activity, free base: $3{\cdot}17 \times 10^5$ counts/min./mgm.) (2), adjusted to pH 7·4, were administered by intraperitoneal injection to Wistar strain Rats 1 (53 gm.) and 2 (109 gm.), respectively. In another series of experiments, Rat 3 (56 gm.) was given by intraperitoneal injection 1 ml. of an aqueous solution of 5·5 mgm. of the antihistamine Neohetramine hydrochloride[3a] and 9·5 mgm. of radiohistamine (specific activity, free base: $1{\cdot}04 \times 10^5$ counts/min./mgm.). This was followed by two administrations of 5·5 mgm. each of the antihistamine, at eight-hour intervals, after the first injection. Rat 4 (55 gm.) was similarly treated, except that three 4 mgm. doses of Neo-Antergan maleate[3b] were administered instead of the Neohetramine.

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[3] *The antihistamines were kindly supplied by: (a) John Wyeth and Brother (Canada) Limited (Walkerville, Ont.); and (b) Poulenc Limitée (Montreal, Que.).*

Collection of Urine

Each animal once injected was placed in a glass metabolism cage for 24 hr. The urine excreted during this period, combined with the urinary bladder content, was diluted to 100 ml. with water. Ten milliliter aliquots of each urine solution were then concentrated by evaporation to 1 ml.

Paper Chromatography of Urine and Radioactive Assays of Paper Chromatograms

Duplicate one-dimensional paper chromatograms were prepared for each concentrated urine solution. Volumes ranging between 15 and 50 microliters of the urine solutions were deposited on No. 1 Whatman filter papers. The chromatographic solvent system employed was *n*-butanol: 95% ethanol: concentrated ammonium hydroxide (8:1:3) (1). One chromatogram of each set was sprayed with diazotized sulphanilic acid (DSA) as coloring reagent (3). Imidazole compounds appeared as red spots.

The duplicate chromatogram of each set (untreated with DSA reagent) was cut into segments 2 cm. wide by 1 cm. high. Each segment was counted under a Geiger–Mueller tube and correction was made for background only. The net counts registered per minute for each segment were plotted against its position in centimeters on the paper chromatogram. The radioactive peaks could thus be located and related to the R_f values of the colored spots on a corresponding chromatogram.

Isotope Dilution Assays of Urine

Isotope dilution assays were carried out on aliquots of urine solutions for histamine, imidazoleacetic acid, and acetylhistamine.

1. *Histamine*

To a 10 ml. aliquot of urine was added as carrier 100 mgm. of histamine dihydrochloride. Histamine was precipitated from the solution by the addition of 0·86 gm. of *o*-dichlorobenzenesulphonic acid dihydrate (4). The solution was warmed on a steam bath and the histamine bis(*o*-dichlorobenzenesulphonate) was allowed to crystallize at room temperature. The histamine derivative was recrystallized from hot water to constant radioactivity.

2. *Imidazoleacetic Acid*

To a 10 ml. aliquot of urine was added as carrier 100 mgm. of imidazoleacetic acid monohydrate. Acetone (30 ml.) was added and imidazoleacetic acid monohydrate precipitated upon cooling. The product was recrystallized to constant radioactivity under the same conditions.

3. *Acetylhistamine*

To a 5 ml. aliquot of urine was added 100 mgm. of histamine dihydrochloride and 5 ml. of 5 *N* hydrochloric acid. The solution was heated for one hour in a boiling water bath under an atmosphere of nitrogen. The hydrolyzed urine was evaporated to dryness *in vacuo* and the residue was dissolved in 5 ml. of

water. The aqueous solution was then treated in a similar manner as described above for histamine precipitation. Acetylhistamine was thus determined by the increase in the radioactivity of the histamine.

Results and Discussion

Since the work of several authors has led to the characterization of the urinary products arising from the metabolism of histamine in the intact rat, we therefore considered that the urinary pattern of these metabolites might be a good criterion for studying the effect of antihistaminic substances. Results obtained by the application of paper chromatographic and isotope dilution techniques are hereby presented.

1. *Paper Chromatography*

Treatment with DSA reagent of paper chromatograms of the urine of Rats 1 and 2 revealed two red spots due to imidazoleacetic acid (R_f 0·19) and histamine (R_f 0·79). However, the radiochromatograms showed that the urine contained three radioactive substances (Fig. 1), peaks 2 and 3 corresponding to imidazoleacetic acid and histamine respectively (2). The nature of the substance at peak 1 has not been definitely established by us, however, it may well be imidazoleacetic acid riboside, recently described by Karjala (5) and Tabor and Hayaishi (8). It may be interesting to mention that some chromatograms treated with DSA reagent showed a faint red spot

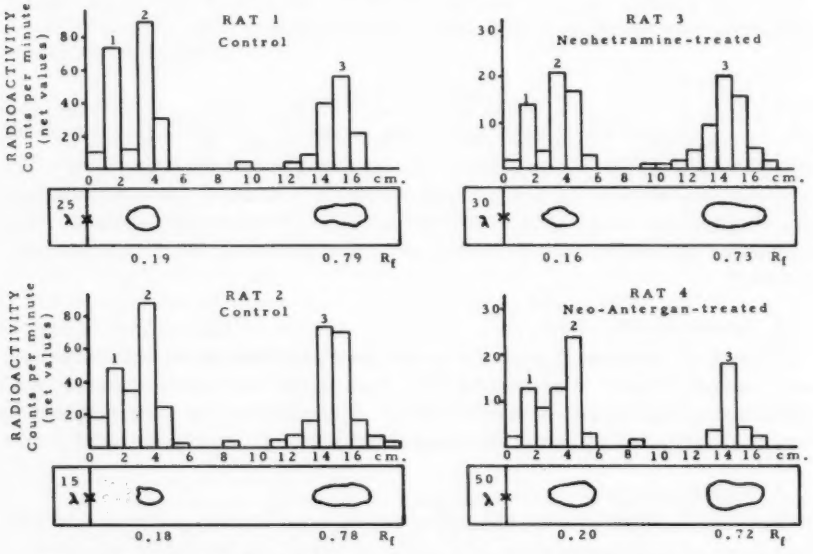

FIG. 1. Distribution of radioactivity on typical paper chromatograms of the urine of rats. The nature of the compound at peak 1 has not been established. Peaks 2 and 3 correspond to imidazoleacetic acid and histamine.

at the position of radioactive peak 1 (R_f 0·08), while on others no coloration was observed. Whatever the nature of the compound at peak 1 may be, the conclusions of the present work will remain unaffected.

The chromatographic urinary pattern of the products of radiohistamine metabolism being known, a comparison can now be made with that of the urine of Rats 3 and 4 which received the antihistaminic substances Neohetramine and Neo-Antergan respectively. The typical urine radiochromatograms presented in Fig. 1 reveal that the imidazole compounds found in the urine of Rats 3 and 4 treated with the antihistamines, Neohetramine and Neo-Antergan, are exactly the same as those excreted by the untreated animals. Moreover if we consider the *relative distribution* of the radioactivity on each paper chromatogram, we find that there is no marked variation between the different chromatograms.

2. *Isotope Dilution*

In Table I, we present the results of the isotope dilution assays of imidazoleacetic acid and histamine, as per cent of the total radioactivity in the urine. Imidazoleacetic acid accounted for 35 to 40% of the urinary radioactivity for both the antihistamine-treated and untreated rats. Similarly, 30 to 40% of the urinary radioactivity of both groups was due to histamine. Acetylhistamine was determined in the urine of Rats 1 and 4 only, and in both cases this histamine conjugate accounted for about two per cent of the total radioactivity in the urine. The carrier technique has therefore shown that antihistamine treatment does not cause any significant change in the excretion of these imidazoles. It may be interesting to mention that the amount of radiocarbon excreted in the respiratory carbon dioxide was found to be negligible in the antihistamine-treated rats, just as it was in the untreated animals (2).

Thus the results of the isotope dilution assays, as well as those of the chromatographic analysis, indicate that Neohetramine and Neo-Antergan treatments do not modify to any measurable extent the urinary pattern of the histamine metabolites in the rat. A similar observation was made by Schayer (7) after he injected a mouse with pyribenzamine.

TABLE I

ISOTOPE DILUTION ASSAYS OF IMIDAZOLEACETIC ACID AND HISTAMINE IN URINE

Rat	Antihistamine injected	Per cent of total radioactivity in urine	
		Imidazoleacetic acid	Histamine
1	None	36	30
2	None	38	38
3	Neohetramine	39	41
4	Neo-Antergan	38	32

Evidently, the action of these antihistaminic substances in the intact rat cannot be attributed to their functioning as inhibitors of histamine metabolizing enzymes.

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